ECOPHYSIOLOGICAL ASPECTS OF *BACOPA MONNIERI* (L.) PENNELL

Thesis Submitted to the University of Calicut
For the Degree of
DOCTOR OF PHILOSOPHY IN BOTANY

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2007

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CERTIFICATE

Certified that the thesis entitled "**ECOPHYSIOLOGICAL ASPECTS OF *BACOPA MONNIERI* (L.) PENNELL**" submitted by **HUSSAIN K.** in partial fulfillment for the Degree of **DOCTOR OF PHILOSOPHY** in Botany, University of Calicut, is a bonafide record of the research work undertaken by him in this Department under my supervision during the period 2001-2007 and that no part of it has been submitted before, for the award of any degree.

Dr. NABEESA SALIM

DECLARATION

I hereby declare that the thesis entitled "**ECOPHYSIOLOGICAL ASPECTS OF *BACOPA MONNIERI* (L.) PENNELL**" submitted by me for the Degree of **DOCTOR OF PHILOSOPHY** in the Faculty of Science, Plant Physiology and Biochemistry Division, University of Calicut has not been submitted for the award of any other degree.

HUSSAIN K.

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ACKNOWLEDGEMENTS

With great pleasure I express my deep sense of indebtedness and heartfelt gratitude to my guide ***Dr. Nabeesa Salim****, Reader, Department of Botany, University of Calicut for her guidance, co-operation and encouragement rendered throughout the course of the project work without which this would not have been fulfilled.*

I extend my gratitude to ***Dr. M. Sivadasan****, Professor and Head, Department of Botany, University of Calicut for facilities provided.*

My sincere thanks are due to ***Dr. P.V. Madhusoodanan,*** *Professor and former HOD, Department of Botany University of Calicut.* ***Dr. K.M. Jayaram****, Lecturer, Dept. of Botany University of Calicut and* ***Dr. K.M. Khaleel****, Dept. of Botany, Sir Syed College, Thaliparamba, Kannur, for their interest in the investigation.*

The help rendered by ***Dr. Jojo Joseph****, Lecturer, Department of Botany, Devagiri College, Calicut is gratefully acknowledged.*

I am grateful to ***Dr. C. K. Nair****, Principal Scientist, CEPC Kollam,* ***Dr. A.D. Mercy****, Associate Professor, Veterinary College, Mannutty, Thrissur,* ***Dr. K.T. Vijaya Madhavan****, Former HOD, Dept. of Zoology, Devagiri College, Calicut, for the experimental facilities and suggestions given.*

I acknowledge my sincere thanks to ***Dr. K.K. Sahadevan,*** *GVHSS Chelary and* ***Dr. S. Sheela****, Lecturer, Madappally Govt. College, Vatakara, for their help.*

I am indebted to all teaching and non-teaching staff of Botany, Zoology, Biotechnology, Chemistry, Life Sciences, Health Sciences, Physics and Instrumentation Depts. of Calicut University for their help throughout the laboratory work of the project.

I convey thanks to all research scholars and M.Phil. students of Dept. of Botany, University of Calicut for their co-operation.

I express my gratitude to ***Adv. P.A. Salim*** *for his encouragement throughout the period of the investigation.*

My sincere thanks are also due to the staff of Bina Photostats for DTP and graphic works of the final thesis.

I express gratitude to ***Mr. C. Kunjali****, Principal,* ***V. Kunhimoideen Kutty****, Manager and other colleagues and staff of H.M.Y.H.S.S. Manjeri.*

The co-operation and encouragement rendered by my parents and family members are gratefully acknowledged.

HUSSAIN K.

Dedicated to

The Almighty - My Strength and Motive

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INTRODUCTION

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Plant ecophysiology describes the responses of plants under prevailing conditions and provides a causal analysis of physiological mechanisms and the corresponding ecological factors. Ecophysiology occupies a frontier between physiology and evolution and this science deals with the interaction between plants and their environment. Ecophysiological research applies not only to overall responses to variations in the complex environment in the natural condition, but also to isolated effects to each of the factors included and their interactions. For the study of those aspects, laboratory experimentation is as the whole, more appropriate and there must be a systematic effort to coordinate and relate laboratory experiments and full observations (Leclerc, 2003). In recent years, many studies in plant ecophysiology concern how whole plant responses to changing environmental conditions.

Heavy metal pollution of the environment due to geogenic, anthropogenic, industrial and agricultural origin represents potential danger for living organisms (Foy *et al.,* 1978; Lepp, 1981; Fitter and Hay, 1983; Prasad, 1997; Orcutt and Nilsen 2000; Cseh, 2002; Pilon-Smits, 2005). According to Salt *et al.,* (1998) and Cobbett and Goldsbrough (2002) soil and water pollution by heavy metals such as Hg, Cd, Pb etc. has been a serious problem in global agricultural productions.

Phytoremediation is a new in-expensive, eco-friendly restoration technology using plants to reduce soil and water pollution due to soluble minerals and metals. According to Leclerc, (2003) in aquatic environment many species accumulate heavy metals and can serve as bio-indicators of cumulative heavy metal pollution. Aquatic plants absorb and accumulate comparatively more quantity of heavy metals from polluted soil and water. According to Pilon-Smits (2005), phytoremediation can be achieved by extraction of metals from polluted soil by planting harvestable plants (Phytoextraction) or by the accumulation of the metals in the root tissue of aquatic plants growing in contaminated water (Rhizofiltration).

Bacopa monnieri (L.) Pennell commonly known as water hyssop and 'Brahmi', a member of Scrophulariaceae family, is a small creeping emergent herb growing naturally in wet soil, shallow water and marshes. This plant has been used in Ayurvedic system of medicine for centuries. Traditionally it was used as a brain tonic to enhance memory development, learning and concentration. The compounds responsible for pharmacological effects of *Bacopa monnieri* include alkaloids, saponins and sterols (Nair, 1987; Anonymous, 2004).

In modern medicine, the Brahmi is well known as a 'nerve tonic' (Nair, 1987). The 'Brahmin' content of the plant body is an important drug in Ayurveda for improvement of intelligence, memory and revitalisation of sensory organs (Sivarajan and Balachandran, 1994). Many products derived from *Bacopa monnieri* are available in the nutraceutical market with content of bacoside A and B (Deepak and Amit, 2004). According to Wohlmuth (2001), Nathan, (2001) and Stough, (2001), Brahmi (*Bacopa monnieri*) is an ayurvedic herb currently enjoying the popularity as 'brain herb' due to its effects on cognitive functions. The plant, plant extract and isolated bacosides (the major active principles), have been extensively investigated in several laboratories for their neuropharmacolgical effects confirming their nootropic action (Martis and Rao, 1992; Stough *et al.*, 2001; Sumathy *et al.,* 2002; Rao *et al.,* 2003; Russo *et al.,* 2003; Deepak and Amit, 2004; Russo and Borrelli, 2005).

Bacopa monnieri plant has been recommended as an agent for phytoremediation (Sinha and Chandra, 1990; Sinha *et al.*, 1996; Sinha, 1999; Yadav *et al.,* 2005). According to those authors, this plant is capable of absorbing and accumulating Hg^{2+} from Hoagland nutrient medium artificially contaminated with $HgCl_2$. Sinha (1999) reported the absorption and accumulation of Copper, Cadmium, Chromium, Manganese and Lead in the root and shoot system and the quantity accumulated is depending on the concentration and duration of treatments with these metals.

Mercury is an industrial heavy metal toxicant causing many phyto - toxic effects. Eventhough angiosperms are not reported as tolerant to Hg, some plants absorb and accumulate considerable quantity of Hg when grown in contaminated soil due to geogenic, anthropogenic and industrial activities (Lepp, 1981; Verkleij, 1993; Ross, 1994; Orcutt and Nilsen, 2000; Cseh, 2002). Studies on phytotoxic effects of Mercury on plants have been centred on tolerance mechanism, metal accumulation and detoxification methods (Velasco-Alinsug *et al.,* 2005). Nevertheless, no plant has been identified as Hg hyperaccumulators (Henry, 2000; Raskin and Ensley, 2000).

Cadmium is a wide spread pollutant that is known to be one of the most phytotoxic heavy metal (Prasad, 1997; Salt *et al.*, 1998; Orcutt and Nilsen, 2000; Perfus-Barbeoch *et al.*, 2002). Toxic effect of Cd includes cellular damage, denaturing proteins, and displacing of co-factors from a variety of proteins including transcription factors and enzymes (Liao *et al.*, 2002). Phytoremediation of Cadmium has been effectively performed by *Silene vulgaris* (Joop *et al.*, 1994), *Brassica juncea* (David *et al.*, 1995), *Lepidium campestris* (Wenzel and Jockwer, 1999), *Polygonum thunberjii* (Shinmachi *et al.,* 2003), *Potamogeton pectinatus* (Rai *et al.*, 2003), *Brassica juncea* (Ishikawa *et al.*, 2006).

In addition to the well established multipurpose medicinal use, *Bacopa monnieri* has been recommended for phytoremediation due to the hyperaccumulation character (Sinha *et al.*, 1996; Sinha, 1999; Yadav *et al.*, 2005). In this context the fate of the plant for medicinal uses should be determined and considered as part of risk assessment because many Ayurvedic preparations containing *B. monnieri* is available in the market and are consumed by humans. Therefore, the dual uses or qualities of *B. monnieri i.e.,* phytoremediation on one hand and medicinal on the other do pose a threat to mankind.

Most of the investigations of phytoremediation are focussed on aquatic weeds and some crop plants. Studies on the responses of medicinal plants in general and aquatic/semi aquatic/emergent medicinal plants in particular to heavy metals like Hg and Cd are very scanty. Moreover, effect of Mercury and Cadmium on aquatic and wetland medicinal plants have not been documented. *B. monnieri* is an ideal plant for this purpose due to the following characters: 1) since *B. monnieri* is a vegetatively propagated plant, cultivation in nutrient solution artificially contaminated with heavy metals can be effectively done, 2) the plant is highly sensitive to soil/water pollution, 3) as the entire plant is used for medicinal purpose, accumulation of toxic metals in root, stem and leaf may highlight the gravity of health hazard due to the entry of these toxic metals to human body.

Hence, the over all objectives of this project are: (1) determination of the concentration of Hg and Cd in which *Bacopa monnieri* plants can tolerate and survive, (2) analysis of phytotoxic effects on growth pattern, tolerance index and stomatal index of the plant, (3) localization of the heavy metals in the root, stem and leaf (4) estimation of dry matter, protein content, protein profile and chlorophylls, (5) comparison of accumulation of Mercury and Cadmium in the root, stem and leaf during 12 days of growth in nutrient medium, (6) bioaccumulation pattern due to different concentrations (dozes) of $HgCl_2$ and $CdCl_2$ during prolonged period of treatments (50 days), (7) assessment of the feasibility of *B. monnieri* for phytoremediation technology and (8) evaluation of the health hazard due to the medicinal use of *B. monnieri* plants by biomagnification experiments.

Preliminary study on *B. monnieri* plants proved the high sensitivity of the plant towards water pollution (un published data). Hence, the present study is extended to examine the accumulation of heavy metal contaminants, which are known to be present in commercially available mineral water, soft drinks, fruit juices and various Brahmi products by culturing rooted propagules in these products or solutions.

Indiscriminate use of polluted area (both soil and water) for large scale cultivation of *Bacopa monnieri* by manufacturers of Ayurvedic medicines and food supplements obviously lead to health hazard because of the heavy metal bioaccumulation potential of these plants. Hence, some remedial measures to check or control the absorption and translocation of the toxic heavy metals are proposed to be recommended by analysing the mode of absorption of heavy metals by changing appropriate pH and/or by adding some antagonistic substances to the growth medium/soil.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Plants are continually exposed to toxic chemicals including heavy metals. Availability and toxicity of heavy metals, responses and adaptive strategies of plants to metal toxicity and phytoremediation technology have been extensively discussed and excellently reviewed by several authors (Foy *et al.,*1978; Barcelo and Poschenrieder, 1990; Rauser, 1990; Steffens, 1990; Fernandes and Henriques, 1991; Mc Neilly, 1994; Prasad, 1997; Salt *et al.*, 1998; Bhomik and Sharma, 1999; Orcutt and Nilsen, 2000; Cseh, 2002; Fodor, 2002; Zimmels, 2004; Pilon–Smits, 2005; Weber *et al.*, 2006 ; Migocka and Klobus, 2007).

Elevated concentrations of both essential and non-essential heavy metals in the soil and water can lead to toxicity symptoms and growth inhibition in most plants (Foy *et al.,* 1978; Lepp, 1981; Grant *et al.,* 1998; Jindal and Kaur, 2000; Hall, 2002). Absorption, translocation and accumulation of heavy metals like Hg^{2+}, Pb^{2+} and Cd^{2+} by plants, reduce qualitative and quantitative productivity of the species and cause a serious health hazard through the food chain to other life forms (Turner, 1994; Barman *et al.,* 1999; Petr *et al.,* 1999; Moreno-Caselles *et al.,* 2000; Axtell *et al.*, 2003; Cobbett, 2003; Stolt *et al.*, 2006).

According to Ho and Sachs (1989) plants develop both a strategy of avoiding uptake of toxic heavy metal ions and ability to synthesize proteins and peptides that can tightly bind and sequester heavy metals. The mechanism that plants use to alleviate heavy metal stress is the synthesis of metal binding polypeptides, the phytochelatins (PC). The apparent function of phytochelatin is to sequester and detoxify excess metal ions. Failure in synthesizing these peptides results in growth inhibition or cell death. The role of phytochelatins in plant metal tolerance has been reviewed by many authors (Tomsett and Thurman, 1988; Rauser, 1990; Steffens, 1990; Robinson *et al.,* 1993; Salt and Rauser, 1995; Grill *et al.*, 2002; Tausz *et al.*, 2004; Callahan *et al.,* 2006).

Plants avoid heavy metals uptake by root exudates to complex metals or by immobilization on cell wall whereas plant tolerance to toxic and excess essential metals is based on a strict regulation of cellular metal homeostasis. (Hall, 2002; Mallick and Rai, 2002; Callahan *et al.*, 2006). Cadmium ion detoxification by thiol peptides resulting in glutathione derived metal complexing phytochelatins, play an important role in plants (Cobbett and Goldsbrough, 2002; Hall, 2002). Heavy metal detoxification is finally achieved by active transport of metal-PC complex into plant cell vacuoles (Salt and Rauser, 1995). Glutathione is the precursor of metal binding protein (Phytochelatin), the synthesis of which is induced by heavy metals and hence involved in heavy metal detoxification (Grill *et al.,* 2002)

According to Tausz *et al.,* (2004) upon exposure to potentially growth inhibiting external concentrations of heavy metal ions, plants respond with the activation of detoxification pathways. The activation can be post translational or transcriptional. Well documented is the activation of phytochelatin synthesis, which occurs upon binding of metal ions or metal GSH complexes to the constitutively expressed enzyme (Vatamaniuk *et al.,* 2000; Maier *et al.,* 2003). An increase in transcript level has been reported in wheat roots (Clemens *et al.*, 1999) and in *Arabidopsis* seedlings after Cd^{2+} treatment (Lee and Korban, 2002).

Different heavy metals at supra optimal concentration, have been shown to inhibit various metabolic process in plants resulting in their reduced growth and development (Bala and Setia, 1990; Davies, 1991; Bernier *et al.,*1993; Lang *et al.,* 1995; Shaw, 1995; Tomar *et al.,*2000). At cellular level metal toxicity causes destruction of enzymes and damage to DNA and also forms the increased production of free radicals (Cseh, 2002).

Understanding how plants accumulate and store metal ions is relevant, to metal nutrition for food and agriculture and metal detoxification mechanism using plants as biological detoxification system. Phytoremediation is a promising method for decontamination of soil and water in the environment (Salt *et al.*, 1998; Reeves and Baker, 1999; Pance *et al.*, 2000; Clemens *et al.*, 2002; Cobbett, 2003; Peuk and Rennenberg, 2005; Pilon-Smits, 2005). Some plants can accumulate remarkable levels of metals 100–1000 fold the levels normally accumulated in most species and such hyperaccumulator species have been identified (Baker and Whiting, 2002). Aquatic plants can remove heavy metal contamination from the surrounding water and soil (Dushenkov *et al.,* 1995; Axtell *et al.*, 2003; Cobbett, 2003; Sersen *et al.,* 2005).

Mercury is discharged into the aquatic environment mainly in the form of elemental mercury (Hg), divalent mercuric ions (Hg^{2+}) and phenylmercuric acetate [$C_6H_5Hg(CH_3COO-)$] (Moore and Ramamoorthy, 1984; Driscoll *et al.,* 1994). Mercury-based fungicides such as $HgCl_2$ and phenyl mercuric acetate are extensively used in seed dressing and seed preservation in many countries including India (Maude and Bambridge, 1985; Semu *et al.,* 1985). This may lead to accumulation and quantitative increase of mercury in the top soil.

Mercury has always been reported to be more toxic to plants compared to other heavy metals like Cadmium (Fergusson, 1990; Kneer and Zenk, 1992; Gadallah, 1994; Shaw, 1995), Chromium (Chandra and Garg, 1992; Garg *et al.,* 1994) and Lead (Huang *et al.,* 1987; De Grado *et al.,* 1999; Xiong, 1999; Orcutt and Nilsen, 2000).

Toxicity of mercury has been reported in many plants and in very low concentrations mercury causes hazards to plant growth (Vallee and Ulmer, 1972; Sandmann and Boger, 1983; Kagi and Hapke, 1984; Baker *et al.,* 1985; De *et al.,* 1985). Various forms of growth retardation and physiological changes have been reported in plants by Mercury toxicity (Nag *et al.,* 1980). In *Cyperus rotundus* and *Chloris barbata* root growth inhibition has been reported due to Mercury treatment and the rate of inhibition increased with the increase in concentration of Mercury (Lenka *et al.,* 1993). Maitani *et al.,*(1996) observed a reduction in the relative root elongation of *Rubia tinctorium* in root cultures when treated with 10μM Hg^{2+} whereas relative root elongation registered an enhancement with 100 μM Pb^{2+}.

Mercury enters plants as inorganic forms from the soil or water or from the process of methylation that occurs in plants. According to Woolhouse (1983) mercury after being absorbed from the soil remains deposited mostly in the root tissues. Mercury forms stable complexes with a variety of organic ligands and has exceptional affinity for sulfhydryl groups of proteins (Falchuk *et al.,* 1977; Nath *et al.,* 1993). According to Jain and Puranik (1993), one of the mechanisms by which Mercury exerts its toxic effects is by interaction with essential – SH group of enzymes and structural proteins.

Mercury is an inhibitor of enzymes and proteins in biological systems and all mercurial compounds are highly toxic to plants in general and aquatic plants in particular (Vallee and Ulmer, 1972; De fillippis, 1979; Baker and Walker, 1989; Reed and Gadd, 1990). Brzyska *et al.*, (1991) reported that Mercury inhibits both the soluble and immobilized enzyme activity even at low concentration (0.2 mM) by 20 to 50%.

Phytochelatin is the most abundant class III metallothionein produced in higher plants due to Hg^{2+} exposure (Maitani *et al.,* 1996). Those authors suggested that, since mercury (II) has a linear configuration in co-ordination compounds, PC can effectively protect plants against the Hg^{2+} toxicity. Reduced glutathione (GSH) is the predominant, free thiol present in plants and the concentration of GSH in plant cell is modified by developmental and environmental factors such as heavy metals and cell culture studies have indicated that GSH is the precursor for the synthesis of heavy metal binding phytochelatins (Grill *et al.*, 1987; Rauser, 1987; Scheller *et al.,* 1987; Obata *et al.,* 1994). According to Tukendorf and Rauser (1990) and De Vos *et al.,* (1992) accumulation of phytochelatins is associated with decline in GSH. Jain and Puranik (1993) reported that supply of 0.01 to 0.1mM reduced glutathione to excised greening maize leaf segments prevented the inhibitory effect of Mercury on chlorophyll biosynthesis and the supply of other thiols such as dithiothreitol, cysteine and mercaptoethanol also reduced the inhibition of chlorophyll formation by Mercury. Increased content of malondialdehyde in plants treated with Hg was reported due to the inhibition of the enzymes of photosynthetic carbon reduction (PCR) cycle (Sheoran *et al.*, 1990; Van Assche and Clijsters, 1990; Shaw, 1995). Mercury has got direct effect on the photosynthetic electron transport (Prasad *et al.,* 1991) causing generation of singlet oxygen and super oxide radicals than the Cd.

Shaw (1995) reported that *Phaseolus aureus* when exposed to $HgCl_2$ showed enhanced lipid peroxidation and activities of antioxidative enzymes (catalase guaiacol peroxidase and ascorbate peroxidase).

Mishra and Choudhuri (1996) investigated the membrane damage caused by Pb^{2+} and Hg^{2+} in two rice cultivars and observed an increase in the activity of lipogenase and malondialdehyde content due to treatment with both the heavy metals. Mercury increased the activity of peroxidase and the level of H_2O_2 while decreased the activities of SOD and catalase. An increase in leakage of electrolytes was also observed. The authors concluded that, Hg^{2+} and Pb^{2+} caused membrane damage in *Oryza sativa* was mediated by reactive oxygen species and hydrogen peroxide induced by these metals.

Based on the detailed study on the effect of mercury on photosystem II sub-membrane fraction of barley leaves (*Hordeum vulgare*), Bernier *et al.*, (1993) reported that Mercury is an environmental contaminant that strongly inhibits photosynthetic electron transport. Photosystem II being the most sensitive target, oxygen evolution was strongly inhibited and chlorophyll fluorescence was severely quenched by Mercury. Prasad and Prasad (1987), Jain and Puranik (1993) and Prasad (1997) have reported decline of chlorophyll content due to Mercury toxicity.

Studies on effect of mercury on growth of *Phaseolus vulgaris* seedlings revealed that Mercury inhibited root and hypocotyle elongation and decreased chlorophyll content (Parmar *et al.*, 2002). Peroxidase activity was higher in mercury treated seedlings compared to control. According to Parmar and Chanda (2005), not only peroxidase activity but IAA oxidase activity also was increased due to the treatment with

Mercury and these enzyme activities are considered as one of the defence mechanisms in response to heavy metal toxicity.

As mentioned earlier, the major impact of Mercury is that it has a high affinity for sulfhydryl group and thus can inactivate many proteins and enzyme systems in plants. Mercury appears to be easily mobilized in plants, hence it can be accumulated in plant parts that could be consumed by humans or other animals. Consequently, like other heavy metals that can be accumulated in plants, it is a concern related to animal and human health (Orcutt and Nilsen, 2000).

Mercury binding peptides from roots, stems and leaves of Hg-treated *Chromolaena odorata* were isolated and partially characterised using RP-HPLC and ESI-MS (Valasco-Alinsug *et al.*, 2005). The plant's ability to accumulate and sequester Hg ions was primarily attributed to the production of Hg-binding peptides.

Soil is contaminated with Cadmium since phosphate fertilizers contain Cd about 70–150 mg kg^{-1} (Andersoon and Siman, 1991). Cadmium ions are comparatively more mobile and its complexes are less stable, so Cd^{2+} occurs as free ions and have significant toxic effects (Verkleij and Schat, 1990; Cseh, 2002; Reid *et al.,* 2003).

According to Pinot *et al.,* (2000) Cadmium ions occur naturally in soil and are a major environmental pollutant and uptake by plants is the main source of Cadmium accumulation in food.

Cadmium in water bodies originate from geogenic and anthropogenic sources such as industrial effluents, agricultural and sewage sludges. Aquatic plants have the ability to accumulate large quantity of Cd and hence these plants could be used for phytoremediation (Dunbabin and Bowner, 1992; Zayed *et al.*, 1998; Rai *et al.*, 1995; 2003).

Cadmium is a highly toxic heavy metal the presence of which in the environment is essentially due to human activities. But this trace element with potential causes human health problems. Cadmium reaches human through food consumption due to high intake of food contaminated with Cd (Wagner, 1993). General symptoms of Cd phytotoxicity are chlorosis, growth retardation, respiratory and nitrogen metabolic changes and low biomass production (Sanita-di-Toppi and Gabbrielli, 1999), inhibition of chlorophyll synthesis and non cyclic photophosphorylation (Orcutt and Nilsen, 2000) and changes in plant water relations (Seregin and Ivanov, 2001; Perfus–Barbeoch *et al.*, 2002).

The inhibitory effect of Cd is generally dependent on the age of the plant; the younger the plant, the greater the damage (Shaw and Rout, 1998). Cadmium accumulates mostly in root cell wall and inhibits root elongation. Cadmium stress facilitates the suberisation of endodermis and exodermis and the endodermis is extended towards the root tip shortening the zone of high water uptake capacity. Heavy metals induce incorporation of lignin and more lignification increases rigidity of the cell wall and inhibit root elongation resulting in decrease of water uptake culminating in the manifestation of growth retardation.

Cadmium is absorbed by roots *via* essential metal trancripters (Cohen *et al.*, 1998; Pance *et al.*, 2000) and after prolonged exposure Cd^{2+} ions are translocated to the shoot and cause growth retardation and carbon assimilation (Krupa and Baszynski, 1995) and these ions induce oxidative stress in plants. Absorption and translocation of Cd varies from plant to plant. Genetic variations exist in accumulation rate of Cd in different parts of the same plant (Stolt *et al.,* 2006). Those authors suggested that one genotype accumulate maximum Cd in the shoot and in the other genotype maximum Cd was accumulated in the grains and both these observations were not clearly correlated with the concentration of Cd in the soil. Stolt *et al.* (2003) reported that transport of Cd from the root to the shoot varies between genotypes of wheat plants.

Cadmium exerts its inhibitory effect in plant cells by binding to specific groups of proteins inhibiting their normal function especially channel proteins of membranes and so uptake of many essential elements may be disturbed (Cseh, 2002). Synthesis of Cd induced sulfhydryl-rich peptides (Phytochelatins) have been described as effective chelators of detoxification of this metal. Meuwly *et al.*, (1995) reported the occurrence of three families of thiol peptides induced by Cd in maize. According to Rauser (1995) and Zenk (1996) PCs are rapidly induced in cells and tissues exposed to Cd in many plants.

Cadmium induces formation of free radicals and reactive oxygen species promoting oxidative stress (Stroinski, 1999) resulting in inactivation of antioxidant enzymes. According to Shaw (1995) *Phaseolus aureus* when exposed to $Cd(NO_3)_2$ showed enhanced lipid peroxidation and activities of antioxidative enzymes (Catalase guaiacol peroxidase and ascorbate peroxidase). Oxidative stress such as disturbances in photosynthesis, nitrogen and sulfur metabolism following exposure to high Cd^{2+} (Sanita-di-Toppi and Gabbrielli, 1999) may be an indirect effect of a depletion of reduced glutathione which is involved in the synthesis of phytochelatins (De Vos *et al.,* 1992). Another potential cause of Cd toxicity is the high affinity of Cd^{2+} ions to functional groups of biological molecules in particular SH groups, O and N-containing groups. The binding can render the molecules inactive. Cd^{2+} ions are hypothesised to replace metal cofactors such as Zn^{2+} ions from proteins or compete with Ca^{2+} for binding to Ca^{2+} binding proteins (Stohs and Bagchi, 1995).

Cadmium inhibits transport of Fe ions into the shoot resulting in Fe deficiency symptoms like chlorosis in young leaves. Cohen *et al.,* (1998) suggested that Fe deficiency induces uptake of more Cd in pea seedlings and proposed that a divalent cation transporter may be induced which can speed up the up take of Cd^{2+} and Zn^{2+}. However, the stimulation of Cd up take is only in the roots, so more Cd remains in the root and less is transported to the shoot under iron deficiency. Another reason for concentration of Cd in the root is the presence of SH groups on the root surface which can bind Cd covalently.

Cadmium may have antagonistic or synergistic effect on nutrient absorption and translocation (Orcutt and Nilsen, 2000). According to Cseh (2002) Cd is more likely transported through substitution of the other divalent cations limiting their uptake, e.g. Zn. Zinc may be in competition with Cd in the soil and in the nutrient medium. The excess of Zn may decrease the growth inhibition and the inhibition of CO_2 fixation by Cd (Ali *et al.*, 2000), because Zn not only inhibit Cd uptake but also Cd translocation from the root to the shoot.

Cadmium ions may function as a substitute of Ca^{2+}. According to Skorzynska–Polit *et al.,* 1998) the lowering or increasing of Ca concentration alter Cd distribution in bean plants and in the case of low Ca concentration much more Cd is translocated to the shoot compared to high concentration when most of the Cd remained in the root. Eventhough Cd is more mobile, great differences exist between plant species and varieties concerning the root – shoot ratio of Cd distribution (Guo-Yan *et al.*, 1996).

Cadmium ions circulate in the plant i.e., after absorption from the soil/water, it is transported to the phloem too. Since the phytochelatins are stable in alkaline pH values, their Cd complexes may be translocated into the phloem. Cadmium ions are capable of translocating from leaves to the stem which can be increased by Zn^{2+} in essential concentration (Cakmak *et al.*, 2000).

According to Sanita-di-Toppi and Gabbrielli (1999) immobilization of Cd by binding to the cell wall is one of the reasons of Cd hyperaccumulation and PC associated detoxification by sequestering Cd in vacuoles is also another possibility of hyper accumulation. Similar observation were made in *Brassica juncea* (Hale *et al.*, 2001) and *Thlaspi caerulescens* (Hattori *et al.*, 2006), which grow in poorly polluted nutrients solution showed strong accumulation of Cd which is associated with rapid phytochelatin accumulation in the roots.

Bio-availability of Cd to plants depends upon several soil characteristics and upon plant species and among the soil characteristics that determine plant Cd uptake are total and soluble Cadmium concentration, pH and organic matter content (Sauvé *et al.*, 2000; McBride, 2002; Podar *et al.*, 2004). Another factor that is considered to assess human health hazard and/or phytoremediation is substrate heterogeneity. There is evidence that uptake of heavy metal in general and Cadmium in particular is significantly affected by their spatial distribution in soil (Millis *et al.*, 2004).

Effect of different concentrations of Cadmium on *Cannabis sativa* cultivated in soil culture artificially contaminated with Cd at pH 4.4 and pH 5.5 revealed that growth of *C. sativa* in high concentrations of Cd resulted in significant reduction of biomass production (Linger *et al.*, 2005).

Mobility of Cd^{2+} in potato tubers by radio isotopic study using ^{109}Cd, revealed that most of the Cd^{2+} was absorbed by the basal roots and the absorbed ions were rapidly exported to other part of the plant, especially the stem with significant amount appearing in the tubers (Reid *et al.*, 2003). ^{109}Cd applied directly to the leaves showed that Cadmium can be rapidly distributed *via* phloem to all tissues. The results also suggested that unlike Ca^{2+}, Cd^{2+} has high mobility in both xylem and phloem.

Perfus–Barbeoch, (2002) reported that *Arabidopsis thaliana* subjected to Cd stress when grown hydroponically showed that the plasma membrane of guard cell protoplast was permeable to Cd^{2+} ions and those authors concluded that the Ca^{2+} channels of guard cell protoplast membrane are permeable to Cd^{2+} ions and assumed that Cd^{2+} ions are able to mimic Ca^{2+} and enter guard cells through voltage-dependent Ca^{2+} channels. According to Allen *et al.*, (2000) and Blatt (2000), Cadmium has pleotropic effects, one of those being its diffusion in guard cells through Ca^{2+} channels resulting in a perturbation of Ca^{2+} signaling, a key element in guard cell osmoregulation (Taiz and Zeiger, 2002). This Ca^{2+} channel is similar or perhaps the same that recently described AtTPCI expressed in *Arabidopsis* leaf tissue (Furuichi *et al.*, 2001).

Kim *et al.*, (2002) investigated the transport mechanism of Pb^{2+} and Cd^{2+} in rice roots and suggested that since plants do not require Lead and Cadmium for growth, they probably do not have transporters specialized for their uptake. Therefore, heavy metals are likely to enter into plant cells *via* transporters for essential cations. Those authors observed that Ca^{2+} and Mg^{2+} ions inhibit the transport of Pb^{2+} and Cd^{2+} into rice roots suggesting that these heavy metals may enter the root cells *via* transporters that pass Ca^{2+} and Mg^{2+}. Cadmium ions are taken up into plant cells most likely *via* Ca^{2+}, Fe^{2+} and Zn^{2+} uptake systems (Clemens *et al.*, 1998; Connolly *et al.*, 2002; Perfus-Barbeoch *et al.*, 2002).

Weber *et al.*, (2006) investigated the molecular aspects of the mode of Cd toxicity using quantitative PCR and analysed transcriptome changes upon Cd^{2+} exposure in *Arabidopsis thaliana* and *Arabidopsis halleri* which is a hyper tolerant metallophyte. Those authors suggested the involvement of apparently highly specific Cd^{2+} transcriptional response suggesting the existence of a specific Cd^{2+} sensing system in *Arabidopsis*.

Vegetable crops such as Brinjal, Ladies finger and Spinach cultivated by irrigating with sewage and industrial effluent water revealed that heavy metals like Cd, Pb and Ni accumulated in fruits (Jalali *et al.*, 2006). Cadmium ions get translocated to the rice grains causing health hazards to mankind. According to Hattori *et al.*, (2006) chloride ions have the capacity to enhance Cadmium uptake. Cadmium ions are translocated to the grains of *Oryza sativa* through phloem. Studies on the quantitative analysis of Cd concentration in the *O. sativa* revealed that about 91-100% of the Cd in the rice grain was transported through phloem sap. Radioisotope study using ^{109}Cd showed that in rice Cd is translocated to the grains through phloem sap (Tanaka *et al.*, 2007).

Recently a new approach termed phytoremediation, using plants to remove heavy metals from contaminated environment has been emerged (Cunningham and Berti, 1993; Baker *et al.*, 1994; Raskin and Kumar, 1994; Salt *et al.*, 1995). Some plants known as heavy metal hyperaccumulators can extract unusually high content of heavy metals from environment *via* root system and translocate them to the above-ground parts (Baker and Brooks, 1989; Brown *et al.*, 1994; Kumar *et al.*, 1995; Xiong, 1998). According to Kumar *et al.*, (1995) although some plants can accumulate unusually high concentration of heavy metals, their growth may get significantly inhibited.

Phytoremediation is the process or technology involving the use of plants and their associated microbes for environmental clean up (Pilon–Smits, 2005). During the past 10 years, phytoremediation has gained acceptance as a cost-effective, non–invasive, alternative or complementary technology for engineering–based remediation methods (Raskin and Kumar, 1994; Salt *et al.*, 1995; 1998).

Natural elements inclusive of heavy metals come under inorganic pollutants and they cannot be degraded but they can be phytoremediated *via* stabilization or sequestration in harvestable plants. Non-essential heavy metals such as Cd, Cr, Co, Hg, Se, Pb, V etc., can be phyto-remediated (Blaylock and Huang, 2000; Horne, 2000). According to those authors, plants extract pollutants and accumulate more quantities of them in their tissue, followed by harvesting the above ground plant material, remediation can be effective, so this technology is called phytoremediation.

Plants adapted to metal-contaminated ecosystems could be used as indicators for exploration of metals (Books and Malaisse, 1985; Baker and Proctor, 1990; Dickinson *et al.*, 1991; Berthelsen *et al.*, 1995; Kumar *et al.*, 1995).

In a biomonitoring study by Lenka *et al.*, (1993) two mercury tolerant grasses *Chloris barbata* and *Cyperus rotundus* were isolated from a Mercury polluted locality near a chlor-alkali Plant, where Mercury contamination was as high as 557mg kg^{-1} soil and according to them tolerance to Hg was more in *C. barbata* than *C. rotundus*.

Copper and Cadmium accumulation was determined in *Bacopa monnieri* by Sinha and Chandra (1990) and reported that, this plant showed a capability to accumulate both metals in single and mixed metal treatments. Copper accumulation was stimulated by the presence of Cd, whereas uptake of Cd was inhibited by Cu. Those authors suggested that *B. monnieri* showed high concentration factors of both metals and recommended the possibility of using *B. monnieri* for mitigating Cu and Cd pollution in the aquatic environment.

The uptake and toxicity of Copper and Chromium were studied in single and combined metal treatments in the roots and shoots of *Bacopa monnieri* and *Scirpus lacustris* (Gupta *et al.*, 1994; Yadav *et al.,* 2005). According to those authors the effect was concentration and duration dependent and accumulation being greater in the roots. In combined metal treatments uptake of Cr was increased in the presence of Cu.

Sinha (1999) investigated the accumulation potential of *B. monnieri* and assessed the effect of Cu, Cd, Pb, Cr and Mn under simulated laboratory condition. The accumulation of the metals by roots and shoots was both concentration and duration dependent. Metal accumulation was considerably higher in fine roots than in the shoots and showed the order Mn>Cr>Cu>Cd>Pb. According to the author, this species may be considered for the amelioration of industrially polluted wetlands experiencing regular flushing of waste waters.

Thlaspi caerulescens, an herbaceous annual has received much attention because it has been known to hyperaccumulate Zn (Baker *et al.*, 1994; Brown *et al.*, 1995; McGrath *et al.*, 1997; Shen *et al.*, 1997; Kupper *et al.*, 1999; Escarre *et al.*, 2000; Pance *et al.*, 2000). *Brassica juncea* also is well known due to its hyperaccumulation of Cd and Zn and hence phytoremediation potential (Kumar *et al.,* 1995; Blaylock *et al.,* 1997; Ebbs and Kochian, 1998; Salt *et al.*, 1995; 1997; Haag-Kerwer *et al.,* 1999; Hale *et al.*, 2001; Hamlin *et al.*, 2003; Podar *et al.*, 2004; Ishikawa *et al.*, 2006; Abe *et al.*, 2006).

The plant species like sharp dock (*Polygonum amphibium*) duck weed (*Lemna minor*), water hyacinth (*Eichhornia crassipes*), water dropwort (*Oenanthe javanica)* and calamus (*Lepironia articulate),* are good candidates for phytoremediation of polluted waters (Wang *et al.*, 2002). *Polygonum amphibium* showed accumulation of Nitrate and Phosphate in its shoots, water hyacinth and duck weed were hyper accumulators of Cadmium. Water dropwort and calamus were hyperaccumulators of Mercury and Lead respectively.

Metal detoxification by using plants as a biological detoxification system for the phytoremediation of metal contamination in the environment is one of the important application of metal nutrition. Heavy metals both essential and non essential to plant growth once enter the plant can be sequestered, stored and detoxified and often accumulated and stored in vacuoles (Clemens *et al.*, 2002). Metal hyperaccumulators have been identified in at least 45 plant families and individual species can accumulate different metal such as Zn, Cd, Cu, Co, Ni, Se and As or particular combination of these (Reeves and Baker, 1999). Hyperaccumul ation of heavy metals in plants implies the mechanism of tolerance and these two characters may be genetically distinguishable in a hyperaccumulated species (Assuncao *et al.*, 2003; Cobbett, 2003).

Aquatic plants can remove heavy metal contamination from the surrounding water. Axtell *et al.*, (2003) reported the ability of *Microspora* (a macro alga) and *Lemna minor* (an aquatic angiosperm plant) to remove soluble Lead and Nickel under various laboratory conditions.

Rai *et al.*, (2003) reported that *Potamogeton pectinatus* L. accumulated significant amounts of Cadmium, the highest of which was observed in plants grown in the nutrient medium containing 200 μM Cadmium. Significant reductions in photosynthetic pigments were observed when plants were exposed to Cadmium for 24-96 hrs.

Potential use of naturally growing aquatic plants like *Azolla californiana*, *Eichhornia crassipes, Elodea canadensis, Hydrocotyle umbellata, Lemna minor, Pistia stratiotes* and *Salvinia natans* for waste water purifications has been reported and practiced (Zimmels *et al.*, 2004). Those authors opined that aquatic plants enhance the removal of pollutants by consuming part of them in the form of plant nutrients.

A study on phytoremediation of Arsenic contaminated ground water collected from South Florida (USA) by growing Chinese Break fern (*Pteris vittata*) showed that this fern is a hyperaccumulator of arsenic and about 30% of Arsenic was absorbed by the plant (Tu *et al.*, 2004).

Chromolaena odorata is one of the most dominant species accumulating highly toxic level of Mercury concentration. Rooted cuttings of *C. odorata* were subjected to Hg treatment and exhibited high uptake in the different Hg treatments at varying length of exposure (Velasco–Alinsug, 2005). Among the three vegetative organs, roots accumulated the highest levels of Hg compared to the stem and leaves. Those authors suggested that upon exposure of *C. odorata* plants to 1.0 and 2.0 μM Hg $(NO_3)_2$ treatments for 0–28 days the plants accumulated as much as 125 mg g^{-1} (dry wt.) Hg in roots, 15.28 mg g^{-1} (dry wt.) Hg in the stems, and 0.80 mg g^{-1} (dry wt.) Hg in the leaves indicating that *C. odorata* has a high potential as a phytoremediation agent of inorganic Mercury.

Sersen *et al.*, (2005) demonstrated that natural zeolite (crystallic aluminosilicates) is capable of capturing heavy metal ions and protect plants from the toxic impacts. Maize plants cultured in Knop's solution contaminated with Cu, Hg and Cd in the presence of natural zeolite showed almost complete protection effect and 100% growth restoration was observed. The heavy metal ions are captured by ion exchange substituting K^+ or Ca^{2+} in the zeolite and the metal content in the root were reduced by 43% for Cu, 50% for Hg and 48% for Cd. According to those authors, maize plants accumulate Cd and this plant could be used for phytoremediation.

The concept of hyperaccumulation was originally introduced to define plants containing > 1000 μg g^{-1} of Ni in the dried plant tissue (Brooks *et al.*, 1977). *Brassica juncea* is well known due to its hyper-accumulation of Cd and Zn and hence phytoremediation potential (Blaylock *et al.*, 1997; Salt *et al.*, 1995; 1997; Kumar *et al.*, 1995; Ebbs and Kochian, 1998; Haag-kerwer *et al.*, 1999; Hamlin *et al.*, 2003; Podar *et al.*, 2004).

Phytoremediation of Cd has been effectively performed by *Brassica juncea* (David *et al.*, 1995), *Thlaspi caerulescens* (Brown *et al.*, 1995) and *Polygonum thunberjii* (Shinmachi *et al.*, 2003). These plants are reported to be hyperaccumulators of Cd. Similarly, weed species such as *Silene vulgaris* (Joop *et al.*, 1994) and *Lepidium campestris* (Wenzel and Jockwer, 1999) have been reported to grow actively in Cd polluted soil and Cd accumulation was noticed.

Thlaspi caerulescens an herbaceous annual plant is considered as an absolute metallophyte (Baker and Walker, 1990) and this plant has an ability to accumulate concentration of Zn in excess of 10,000 μg g^{-1} in the (10 mg g^{-1}) foliar dry matter. Baker *et al.*, (1994) confirmed the hyperaccumulating nature of *T. caerulescens* since shoot accumulate up to 30,100 μg g^{-1} Zn and individual Pb and Cd concentration exceeded 1000 and 250 μg g^{-1} respectively. *Thlaspi caerulescens* has received much attention because it has been known to hyperaccumulate Zn (Baker *et al.,* 1994; Brown *et al.,* 1995; Mc Grath *et al.*, 1997; Shen *et al.*, 1997; Escarre *et al.,* 2000, Pance *et al.,* 2000). According to Escarre *et al.*, (2000) *Thlaspi caerulescens* plants from metalliferous and non-metalliferous soils are interesting materials for phytoremediations of Zn and Cd because these plants are able to accumulate large amount of these metals. However, to optimize the process of phytoextraction, it is necessary to combine the extraction potentialities of both metalliferous and non-metalliferous populations.

Potamogeton pectinatus, a submerged aquatic plant shows the ability to accumulate large amount of Cd and other heavy metals and hence could be used in designing of cost-effective phytoremediation system for clearing of metal polluted water (Rai *et al.*, 1995; 2003; Zayed *et al.,* 1998). Rai *et al.*, (2003) suggested that *P. pectinatus* is an ideal candidate for phytoremediation of Cd polluted water bodies and shows tolerance through increased levels of non-protein thiols, cysteins and carotenoids. However, before using the plant for phytoremediation, it is necessary to study the effect of environmental variables that may be encountered in the field studies.

Linger *et al.*, (2005) studied the effect of different concentration of Cd on *Cannabis sativa* cultivated in soil culture artificially contaminated with Cd at pH 4.4 and pH 5.5 and suggested that roots showed strong resistance to Cd and possessed hyperaccumulation potential (more than 100 mg kg^{-1} Cd in dry tissue).

Although phytoremediation is a promising method for the removal of Cadmium from the soil, plants remove Cd^{2+} from soil very slowly. To increase Cd uptake by plants the effect of application of chloride ions (Cl^-) and decrease in soil pH on the quantity of Cd taken up by plants were investigated in pot experiments using soil collected from paddy fields contaminated with Cd from mining waste water and planting *Hibiscus cannabinus*, *Helianthus annus* and *Sorghum vulgare* (Hattori *et al.*, 2006). The application of Cl^- approximately doubled the quantity of Cd uptake by *Helianthus* and *Hibiscus* compared to the controls.

Ishikawa *et al.*, (2006) reported that *Brassica juncea* has been recognized as a suitable plant for metal phytoremediation from soils with moderately low levels of Cd contamination. But studies on maize, rice and sugarbeet cultured in hydroponics containing high levels of external Cd (1mg g^{-1}) showed that *B. juncea* was less able to accumulate Cd compared to rice and sugarbeet. Maize displayed the lowest metal accumulating species. According to those authors, cultivation of rice in Cd contaminated soil showed significant decrease in soil Cd concentration. But soil Cd reduction was not significant after cultivation of *B. juncea* in Cd contaminated soils. So it was suggested that rice is an eligible plant for phytoremediation of soils contaminated with Cadmium.

According to Yoon *et al.*, (2006) plant's ability to accumulate metals from soils can be estimated using the BCF (Bioconcentration factor), which is defined as the ratio of metal concentration in the root to that in soil. It was reported that the ability of the plant to translocate metals from the roots to the shoots is measured using the TF (Translocation factor), *i.e.*, the metal concentration ratio of the plant shoots to roots. Enrichment or hyperaccumulation occurs when a contaminant taken up by a plant is not degraded rapidly resulting in an accumulation in the plant. The process of phytoremediation generally requires the translocation of heavy metals to the easily harvestable plant parts i.e. shoots. By comparing BCF and TF we can compare the ability of different plants in taking up metals from soil and translocating them to the shoots. Tolerant plants tend to restrict soil-root and root-shoot transfers and therefore have more or less accumulation in their biomass, while hyperaccumulator plants absorb and translocate metals into their above ground biomass. Plants exhibiting TF and BCF values less than one (<1) are unsuitable for phytoremediation (Fitz and Wenzel, 2002).

Phytoremediation potential of native plants under field condition revealed that severe phytotoxicity may act as a powerful force for evolution of tolerant plant populations (Shu *et al.,* 2002; McGrath and Zhao, 2003). Effect of Cd on weed species studied by Abe *et al.*, (2006) showed that most of the weed species growing in the paddy field were accumulators of Cd from the soil and hence can be used for phytoremediation of Cd polluted soil and bioassay for Cd in soil.

There are two mechanisms associated with removal of dissolved metals from water by aquatic plants. The first is a fast metabolism-independent surface adsorption and the second is a slow metabolism dependant cellular uptake (Cho *et al.*, 1994). The first process occurs by diffusion and soluble ions can bind or sorb to the outer cell wall of the biomass and the second is transport of ions to the interior part of the cells.

Different aquatic plants have been found to show varying rates of metal ion accumulation. *Salvinia minima* remove 70-90% of Lead and Zinc in 2 days exposure to concentration of 1-9 mg l^{-1} (Srivastav *et al.*, 1993) and *Eichhornia* was shown to separate As, Cd, Pb and Hg (Chigbo *et al.*, 1982). It has been reported that living plants have the ability to accumulate metals from 50 to 1000 times the concentration of the metal in the water from which it is sorbing (Wang and Wood, 1984). Dushenkov *et al,*. (1995) showed that aquatic plant roots could absorb 50% of 500 mg l^{-1} Pb in 45 hrs. This type of bioaccumulation provides a rapid and a high degree of separation and it is inexpensive and eco-friendly and energy saving compared to physical or chemical means of heavy metal removal.

Aquatic plants particularly submerged species are used for the removal of heavy metals like Cu, Cd, Cr, Fe, Mn and Pb from contaminated water and/soil (Guilizzoni, 1991; Rai *et al.*, 1995; Sinha *et al.*, 1997; 2002). Bioaccumulation and effects of Cd, Cr, Cu, Fe, Mn and Hg on submerged plants *Hydrilla verticellata* and *Bacopa monnieri* have been studied by several authors (Sinha *et al.*, 1993; 1997; Rai *et al.*, 1995; Gupta *et al.*, 1998; Sinha, 1999; Sinha and Pandey, 2003). According to Sinha and Pandey (2003) submerged plants like *Hydrilla* possess significant potential to bioaccumulate Ni and accumulation rate and quantity was found to increase proportional to the concentration of metals present with media as well as the period of exposure. Bioaccumulation of heavy metals during repeated exposure to contaminated medium was reported in submerged plant *Najas indica* (Sinha *et al.*, 2003). A study to examine the ability of *Lemna minor* an aquatic plant (Axtell *et al.*, 2003) to remove soluble Pb and Ni under laboratory condition showed 76% of Pb and 82% of Ni removal. No synergistic or antagonistic effect was noted in the multiple metal experiments.

Plants used for phytoremediation enhance the removal of pollutants by consuming part of them in the form of plant nutrients. According to Zimmels *et al.*, (2004) aquatic plants such as *Eichhornia, Pistia, Lemna* and *Salvinia* which are having high efficiency in removing metals and minerals from contaminated water are used for improving river water quality. So the aquatic plant system offers an eco-friendly and cost-effective technology for treatment of urban and agricultural waste water.

Phragmites australis, a perennial emergent aquatic plant when exposed to $CdSO_4$ exhibited high detoxification potential due to phytochelatins synthesis and stimulation of antioxidant systems in spite of high accumulation of Cd in root system (Ederli *et al.*, 2004).

Phytoremediation of Chromium polluted soil and water can be done with harvestable plants (Phytoextraction) and by accumulation in the root tissue of aquatic plants by growing in contaminated water (Rhizofiltration). Yadav *et al.,* (2005) reported phytoextraction of Chromium from contaminated soil by *Scirpus lacustris, Phragmites karka* and *Bacopa monnieri.*

At cellular level, heavy metals toxicity may result in destruction of enzymes and damage to DNA and increased production of oxygen radicals. To maintain a homeostasis within the cell, plants have to keep heavy metal concentration at an optimal, sub-toxic level. For this purpose detoxification process occur by sequestration of heavy metal ions in the vacuoles. Studies on biochemical characterization of systems removing heavy metals to vacuoles provided evidences for the existence of an antiport mechanism for Cd/H and Cu/H ions exchange in the plasma membrane of cucumber root cells (Burzynski *et al.*, 2005). For this secondary transport, antiport systems have been reported for many heavy metals like Cd, Cu, Mn, Ni and Pb (Burzynski *et al.*, 2005; Migocka and Klobus, 2007). Those authors further suggested that the transport of heavy metals in plasma membrane of cucumber roots take place *via* antiport systems utilizing a proton motive force as the source of energy and treatment with Cd and Ni markedly induced antiport activities of Cd, Mn, Ni and Pb (Migocka and Klobus, 2007).

Several studies on toxicity of heavy metals like Mercury and Cadmium, causing serious health hazards have been reported since humans are exposed to various forms of toxic metals like Hg and Cd (Davis *et al.*, 1997). Biomagnifications of methyl Mercury reaching humans through fish, which were cultured in lakes, contaminated with methyl Mercury has been reported by Campbell *et al.,* (2006).

Gochfeld (1997) reported that mice are susceptible to toxic effects of Cd and to accumulate in the liver, which caused lesions in endothelial cells. The author also reported that accumulation of Mercury in the kidney of rats causing damages to the proximal tubular cells. According to this author, bone of rats was reported as a reservoir of divalent cations like Cd^{2+} and Hg^{2+}. Kramarova (2005) also reported accumulation of Cd in the liver and kidney of mouse and deer exposed to contamination of those metals. Karen Kidd (2005) suggested that fishes, fish-eating wild life and man get Mercury and the methyl Mercury absorbed very slowly and get accumulated in kidney which is considered as a serious health hazard.

MATERIALS AND METHODS

Hussain K. “Ecophysiological aspects of bacopa monnieri (l.) pennell” Thesis.
Department of Botany, University of Calicut, 2007

MATERIALS AND METHODS

1. PLANT MATERIAL

Bacopa monnieri (L.) Pennell cuttings were collected from different regions of Malappuram District. Cement pots half filled with potting mixture (soil:sand:cowdung 1:1:1) and flooded with tap water were used for cultivation. Ten cuttings were planted in each pot and maintained under green house condition. Growth performances were observed and most profusely growing plants were selected for experiments. Continuous propagations of plants were done throughout the period of experimentation by re-planting 2-3 times in a year. Healthy cuttings of length 7-8 cms consisting of 5-6 nodes were selected for experiments.

2. ROOTING

The cuttings selected as described above were placed in distilled water and kept under open air condition for rooting. Rooted propagules were used for experiments of heavy metal treatments.

3. CHEMICALS

Either AR or GR grade chemicals were purchased from MERCK/ BDH,SRL and/or Glaxo Laboratories. Some standards and rare chemicals were purchased from Sigma Chemical Company, St. Louis, USA.

4. CONTAINERS FOR CULTURE

Good quality plastic trays of size 12 x 8.5 cm and having a depth of 3 cm were used for the preparation of hydroponics system. Plastic twines of diameter 1mm were tied inter weaving length wise and breadth wise the trays forming a mesh to provide mechanical support to the propagules. The gap provided underneath facilitates room for growth of the root system.

5. COMPOSITION AND PREPARATION OF NUTRIENT SOLUTION

Hoagland solution (1950) modified after Epstein (1972) as described by Taiz and Zeiger (1991) was employed in the present study. Composition of the modified solution is shown in Table I. Stock solution of each nutrient was prepared separately and appropriate volumes were mixed together to make up nutrient solution of final volume and concentration. pH of the solution was adjusted to 6.8 using 1N HCl or NaOH as the case may be.

Table I: Composition of modified Hoagland nutrient solution employed in the present investigation

Compound		Concentration of stock solution	Concentration of stock solution	Volume of stock solution per litre of final solution
		mM	gL^{-1}	ml
Macro nutrients	KNO_3	1000	101.10	6.0
	$Ca(NO_3)_2\ 4H_2O$	1000	236.16	4.0
	$NH_4H_2PO_4$	1000	115.08	2.0
	$MgSO_4\ 7H_2O$	1000	246.49	1.0
Micro nutrients	KCl	25	1.864	2.0
	H_3BO_3	12.5	0.773	
	$MnSO_4H_2O$	1.0	1.169	
	$ZnSO_4\ 7H_2O$	1.0	0.288	
	$CuSO_4\ 5H_2O$	0.25	0.062	
	H_2MoO_4	0.25	0.04	
	Fe EDTA	53.7	30.0	0.3

Adopted from Taiz and Zeiger (1991)

6. TREATMENT WITH MERCURIC CHLORIDE

Ten mM stock solution of $HgCl_2$ was prepared by dissolving 27.15mg of $HgCl_2$ in distilled water and required dilutions were made in Hoagland nutrient medium for culturing of rooted propagules.

Three concentrations of $HgCl_2$ (2, 5 μM and 10 μM) were selected and based on a preliminary study conducted, in which almost 50% growth retardation was taken as the criterion for determining the degree of toxicity.

7. TREATMENT WITH CADMIUM CHLORIDE

Ten mM stock solution of $CdCl_2$ was also prepared by dissolving 18.33 mg of $CdCl_2$ in distilled water and required dilutions were made in Hoagland solution

Preliminary study was done to select the optimum concentrations from 10, 20 and 30 μM $CdCl_2$ and found that 20 μM and 30 μM solutions showed almost 50% growth retardation. So 20 and 30 μM concentrations were selected for all experiments.

8. PREPARATION OF SOLUTION FOR HEAVY METAL TREATMENT

Five μM and 10 μM solutions of $HgCl_2$ and 20 μM and 30 μM solutions of $CdCl_2$ were prepared by proper dilution of the stock solution with Hoagland nutrient medium and 200 mls of each solution was taken in each separate container. Rooted propagules prepared as described earlier were planted in these culture media by inserting the individual propagules, without disturbing the fresh roots, into the container. Eight numbers of propagules were planted in each container and at least a minimum of five containers were used for each treatment. Propagules cultured in Hoagland nutrient solution without any heavy metal salts served as the control.

9. SAMPLING

Random sampling was done by collecting plants from all replicate containers of culture at an interval of 2 days up to 12 days. Propagules collected were cut in to root, stem and leaf and were used for various analyses.

10. MORPHOLOGICAL MEASUREMENTS

Growth of rooted propagules was assessed in terms of root length, stem length, fresh weight, and moisture content and dry weight.

10.1. Root and stem length

The sampled propagules were washed in distilled water, blotted and length of root and stem were measured manually, using a graduated scale. Measurements of not less than five propagules were recorded each time.

10.2. Secondary roots

Secondary roots initiated at each interval were noted.

10.3. Tolerance Index (TI) percentage

Root Tolerance Index percentage was calculated according to the method of Turner (1994).

$$\text{TI\%} = \frac{\text{Observed value of root length in solution with metal}}{\text{Observed value of root length in solution without metal}} \times 100$$

10.4. Stomatal Studies

Stomatal density on abaxial and adaxial sides of the leaf was counted under a light microscope, preparing impressions using nail polish. Stomatal index was calculated according to the method of Meidner and Mansfield (1968).

$$\text{Stomatal index} = \frac{\text{Number of stomata per unit area}}{\text{Number of stomata per unit area} + \text{number of epidermal cells per unit area}} \times 100$$

10.5. Dry weight percentage determination

Randomly selected propagules from each treatment and control were cut into root, stem and leaf and each tissues were weighed in pre-weighed aluminium foil containers and placed in a hot air oven at 100^0C for 1hr then at 60^0C till the weight became constant. Dry weight percentage was calculated according the following formula

$$\text{Dry weight percentage} = \frac{\text{Fresh weight - Dry weight}}{\text{Fresh weight}} \text{ X } 100$$

11. ANTOMICAL/HISTOCHEMICAL STUDIES

11.1. Anatomical study

Free hand sections of stem (1st and 2^{nd} internodes) were taken and stained with safranin and semipermanant slides were prepared. Sections were observed using Nikon microscope (Model Nikon ECLIPSE E400) and photomicrograph were taken using Nikon Digital Camera (Model DxM 1200F) and image analyser.

11.2. Histochemical study

Samples of roots, stem and leaves of control and treated plants were fixed in FAA, dehydrated through alcohol-TBA series, paraffin infiltrated and embedded (Johansen, 1940). Sections were cut at 10 µthicknesses using Leica Microtome (Model RM2125RT) and stained with safranin and or dithizone for histochemical studies and localisation of Hg/ Cd.

12. PHYSIOLOGICAL AND BIOCHEMICAL STUDIES

12. 1. Estimation of total proteins

Protein content was estimated using Folin-Ciocalteau reagent according to the method of Lowry *et al.*, (1951). Bovine serum abumin (B S A) was used as the standard.

12.1.1. Reagents

A - 2% Sodium carbonate in 0.1N NaOH.

B - 0.5% Copper sulphate in 1% Sodium Potassium Tartrate.

C - Alkaline Copper solution 50 ml of reagent A is mixed with 1 ml of reagent B.

D - Folin-Ciocalteau reagent. The concentrated reagent was diluted to 1N.

12.1.2. Extraction

Two hundred mg of fresh tissue from the randomised samples of propagules of each treatment and control were weighed separately. The weighed tissue was ground using mortar and pestle in chilled distilled water. The homogenate was transferred to centrifuge tube and equal volume of 10% TCA was added, mixed well and kept undisturbed in a refrigerator for flocculation.

The precipitated homogenate was centrifuged for 10 minutes, supernatant was decanted off and 2% TCA was added to the residue and again centrifuged, supernatant was decanted off. Later acetone washing was done to remove the pigments. Two washes in 80% acetone and final washing in anhydrous acetone were given. For dissolving protein, 5ml of 0.1N NaOH was added to each centrifuge tubes and boiled for 5 minutes in a water bath, cooled and centrifuged. The supernatant was transferred to test tubes and used for protein estimation.

12.1.3. Estimation

Suitable aliquots were taken in duplicates from each preparation. Then 5ml of reagent C was added, mixed well and kept at room temperature for 10 minutes, and 0.5ml of 1N Folin-Ciocalteu reagent was added to each tube with immediate mixing. The tubes were kept for 30 minutes for colour development. Absorbance (Optical Density) was read at 700nm using a Genesis 20 Spectrophotometer. Bovine serum albumin fraction V powder was used as the standard.

12.2. Electrophoretic study of protein profile

(Polyacrylamide gel electrophoresis, SDS-PAGE).

Protein profile of both Mercury and Cadmium treated and control plants of *Bacopa monnieri* were prepared by SDS- PAGE.

One gram of the fresh tissue collected at each interval was homogenized in 3 ml of Tris-HCl buffer (pH 7.5) after adding 400 mg sucrose in a mortar and pestle by placing it in ice bath. Homogenate was decanted to centrifuge tubes and centrifuged using Plastocraft (Model

ROTA R4R V/FM) refrigerated centrifuge (4^0 C) at 10,000 rpm for 10 minutes. The supernatant was used for PAGE.

The SDS-PAGE was performed in a GENEI gel electrophoretic system according to the method of Laemmli (1970). The protein sample was separated into its polypeptide subunits by adding equal volume of gel loading buffer containing 10% SDS, β- Mercaptoethanol, Glycerol, 0.1% Bromophenol blue and Tris HCl (pH-6.8). The mixture was heated for 2 minutes in boiling water bath and immediately kept in freezer for 1 minute. The sub units were separated electrophoretically using GENEI gel electrophoretic unit in SDS-PAGE slab gel having 10% separating gel and 4% stacking gel (The sample was electrophoresed using Tris-Glycine running buffer of pH 8.3.). The gel after electrophoresis was stained with Coomassie brilliant blue and the bands were compared with markers of known molecular weight by using BIO-RAD (Model Universal Hood II SN 76S/02277).

Composition of the separating gel (10%)

30% Acrylamide / 0.8% Bisacrylamide	3.33 ml
4 x Resolving Gel buffer	2.50 ml
10% SDS	100.00 μl
Double distilled water	4.00 ml
10 %(w/v) Ammonium per sulphate	50.00 μl
TEMED	5.00 μl

Composition of the stacking gel (4%)

30 % Acrylamide / 0.8% Bisacrylamide	670.00 µl
4 x Stacking Gel buffer	1.25 ml
10% SDS	25.00 µl
Double distilled water	3.00 ml
10% (w/v) Ammonium per sulphate	50.00 µl
TEMED	2.50 µl

12.3. Estimation of chlorophylls

Chlorophyll estimation was done according to the method of Arnon (1949) as described by Jayaraman (1981). Fresh leaves of experimentals and control propagules (0.1gm) were washed in distilled water and blotted between sheets of filter papers.

Weighed quantities of fresh leaf tissue were homogenized using clean mortar and pestle in 80% (v/v) acetone. The homogenate was centrifuged at 10000 rpm using tabletop refrigerated centrifuge (Plastocraft, Model ROTA P4R V/FM) for five minutes and the supernatant was collected. The residue was washed again with 80% acetone and centrifuged. This process of washing was repeated until the pellets become colourless. The final volume of the pooled supernatant was noted and absorbance was read at 663nm and 645nm using a Shimadzu (UV-1601) UV-Visible spectrophotometer.

The following formulae were used to calculate chlorophyll contents.

Chlorophyll-a, mg g^{-1} fresh tissue = $12.7\ (A_{663}) - 2.69\ (A_{645}) \times V/\ (1000 \times W)$

Chlorophyll-b, mg g^{-1} fresh tissue = $22.9\ (A_{645}) - 4.68\ (A_{663}) \times V/\ (1000 \times W)$

Total Chlorophylls, mg g^{-1} fresh tissue = $20.2\ (A_{645}) + 8.02\ (A_{663}) \times V/\ (1000 \times W)$

Where A = Absorbance at specific wavelength,
V = Final volume of leaf extract in 80% acetone,
W = Fresh weight of tissue taken.

From the data obtained chlorophyll a/b ratio also was computed.

13. BIOACCUMULATION STUDIES

13.1. Estimation of Mercury and Cadmium

Different plant parts–root, stem and leaf tissues were sampled after 12 days of growth in the culture medium artificially contaminated with different concentrations of $HgCl_2$ and $CdCl_2$ for estimation of the metals accumulated in the plant tissues.

Accumulation of Hg and Cd was also estimated in the samples collected at 10 days of intervals each upto 50 days of growth in nutrient medium to which repeated doses of $HgCl_2$/ $CdCl_2$ were added after each sample cultivation.

Mercury and Cadmium contents in root, stem and leaf tissue were analysed using Atomic Absorption Spectrophotometer (AAS). Samples were prepared according to the method of Allan (1969). Oven dried plant materials were used. Known weight of the sample was wet digested by refluxing in 10ml of nitric acid, and perchloric acid mixed in the ratio of 10:4 until the solution became colourless by using Kjeldahls flasks heated in a sand bath. Then the samples were transferred to standard flasks and made up to 50 ml and kept in screw-capped containers. Mercury and Cadmium content of the residual nutrient medium after treatment also was estimated. Atomic Absorption Spectrophotometer (PERKIN ELMER A Analyst 300) available at Cashew Export and Promotion Council (CEPC), Kollam, was used for estimating Mercury and

Cadmium and other essential and non-essential heavy metals of all samples.

13.2 Effect of pH on the accumulation of Hg/Cd

13.2.1 Control

$HgCl_2/CdCl_2$ was added directly to the distilled water without any pH change.

13.2.2. Acidic pH

Distilled water was used as the medium and adjusted the pH by adding small quantities of 1M NH_4Cl (1-2ml) solution. After adding $HgCl_2/CdCl_2$, pH was noted and recorded. The pH changes are given in Table 15.

13.2.3. Alkaline pH

To distilled water, a small quantity of lime water was added to obtain alkaline pH. After adding $HgCl_2/CdCl_2$, pH changes were noted and are given in Table 15.

14. SENSITIVITY OF *BACOPA MONNIERI* TOWARDS HEAVY METALS

14.1. Natural habitat

Naturally growing plants were collected from different areas of Northern Kerala as mentioned below and heavy metal contents were estimated using Atomic absorption spectrophotometry.

1). Uppala (Kasaragod Dist.) 2). Calicut (Kozhikode Dist.) 3). Kanjikode (Palakkad Dist.) and 4). Mannuthy (Thrissur Dist.).

14.2. Water samples of different locality of Calicut University Campus and nearby areas of Malappuram District

Rooted propagules of *Bacopa monnieri* were cultivated in the following water samples and estimation of heavy metal contents present in the water samples and accumulated in the plants was done.

1) Tap water 2) Well water 3) Bore well water 4) Rain water 5) Sewage water from Calicut City 6) Effluent water of Water Treatment Plant of Calicut University 7) Black stone quarry water from Mankada (Malappuram Dist.) 8) Chaliyar river water (Arekode area) 9) Chaliyar river water (Feroke area) 10) Pond water 11) Paddy field Water and 12) Marine water from Parappanangadi (Malappuram Dist.).

14.3. Different Commercially available mineral water

Rooted propagules were cultivated in the following mineral water samples and estimation of heavy metal contents present in the mineral water samples and accumulated in the plants was done.

1) Aquafina
2) Aquaspring
3) Kingfisher
4) Omkar
5) Soorya
6) Surabhi and
7) Lehar pepsi

14.4. Different Commercially available soft drink samples

Rooted propagules were cultivated in the following soft drinks and estimation of heavy metal contents present in the soft drink samples and accumulated in the plants was done.

1) Coca Cola
2) Pepsi
3) Mirinda
4) Fanta
5) Limca
6) Thums Up
7) Maaza and
8) Sprite.

14.5. Different Commercially available fruit juice samples

Rooted propagules were cultivated in the following bottled/packeted fruit juices and estimation of heavy metal contents present in the fruit juice samples and accumulated in the plants was done.

1) Appy (apple juice)
2) Frooti (mango juice)
3) Rasna (orange juice) and
4) Tang (lemon juice)

14.6. Different Commercially available Brahmi products

Rooted propagules were cultivated in the following Brahmi products and estimation of heavy metal contents present in the Brahmi product samples and accumulated in the plants was done .

1) Brahmi vita
2) Brahmi rich
3) Brahmi plus capsules
4) Jyothish brahmi and
5) Brahmi syrup.

Whenever the concentration of each medium is toxic to growth of the plant, proper dilutions were made with double distilled water. Samples of all media were analysed for the estimation of all heavy metals present. Similarly heavy metal contents accumulated in the plant tissues grown for 4 days each in all media were also analysed using AAS.

15. BIOMAGNIFICATION STUDIES

Experiments for biomagnification study of heavy metals were done in experimental Swiss Albino Mice purchased from Small Animal Breeding Station (SABS) Mannuthy, Thrissur. A known quantity (0.5ml of 20%) of homogenate prepared by grinding plants treated with $HgCl_2/CdCl_2$ was intragastrically administered to three sets of mice contributing 12 numbers each. Three dozes of extract was given at 7 days of interval. Mice without any treatment served as control.

First set of sampling of animals was done on 7th day from one set of animals. Additional doze of 0.5ml extract was administered to the remaining two sets of animals and samples were collected after 7 more days. Third doze of plant extract was given to each set of animals and sampled after 7 more days. So the last samples of animals were given 3 dozes (0th, 7th, and 14th days) of the treatment. Thus three dozes of 4.8 μg Mercury (Total 14.4 μg Hg) as well as three dozes of 33.9 μg cadmium (Total 101.7 μg Cd) were administered.

Control and experimental animals were used to collect blood samples by heart puncturing method and various organs–brain, bone, liver, kidney, muscle, skin and hair were also collected after dissection. Each organ was washed, blotted and dried in hot air oven at 60-65^{0}C. Then the known weight of dry material was used for AAS study after digestion as described earlier.

16. STATISTICAL ANALYSES

All experiments were repeated a minimum of five times and the mean values, standard deviation and standard error were calculated. Mean values ± standard errors are given in tables. Test of significance was done using Fisher's 't' test.

RESULTS

RESULTS

EFFECTS OF $HgCl_2$

1. MORPHOLOGICAL MEASUREMENTS

Root formation (adventitious) was started from first node of the cutting cultured in Hoagland solution (control). Second day onwards root length was increased gradually followed by secondary root initiation. *Bacopa monnieri* showed morphological variations due to the treatment at 2, 5 and 10 µM of $HgCl_2$, from 2nd day onwards. After 4th day of transplantation most important effect noticed was inhibition of new root initiation and elongation of existing roots compared to control (Fig.1). The stunted nature of root was proportional to concentrations *i.e.*, 10 µM $HgCl_2$ showed maximum stunted nature of roots (Fig.1 a,b,c).

1.1. Root length

Treatment of *Bacopa monnieri* with $HgCl_2$ at 2 µM concentration showed a gradual increase of root growth while in 5 µM the gradual but insignificant progress was occurred. At 10 µM concentration resulted in complete inhibition of root growth up to 8th day of treatment and thereafter a significant increase ($P<0.02$) was observed in all treatments. The increase in growth was inversely proportional to increase in treatment concentrations (Table 1, Fig.2) in comparison with control, which showed gradual as well as significant growth in length during the entire period of experiment.

Fig. 1

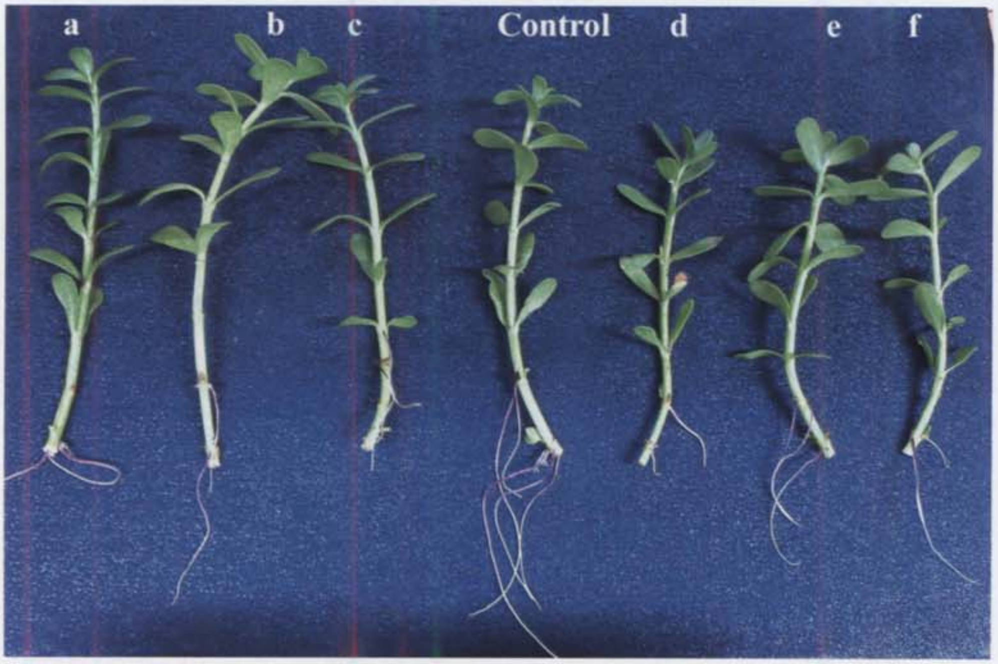


Fig. 1 Effects of $HgCl_2$ and $CdCl_2$ on *Bacopa monnieri*-morphological variations after four days of growth

a - $HgCl_2$ (2μM)
b - $HgCl_2$ (5μM)
c - $HgCl_2$ (10μM)
d - $CdCl_2$ (30 μM)
e - $CdCl_2$ (20 μM)
f - $CdCl_2$ (10 μM)

1.2. Shoot length

In comparison with control, it was observed that plants treated with all above mentioned concentrations of $HgCl_2$ showed only negligible increase in shoot length during 12 days (Table 1, Fig.3).

1.3. Secondary roots

Eventhough secondary root formation was started 2nd day onwards in control plants, secondary root initiation started on 8thday, 10th and 12th days in plants treated with 2, 5 &10 μM $HgCl_2$ respectively (Table 1).

Table: 1 Effect of Mercury on root length, shoot length, and secondary root length in *Bacopa monnieri* during growth.

Tissue	Treatment concentrations ($HgCl_2$)	Interval - Days						
		0	2	4	6	8	10	12
Root length (cm)	Control	1.58 ± 0.28	1.88 ± 0.27	3.25 ± 0.28	4.84 ± 0.30	5.60 ± 0.31	6.32 ± 0.31	7.95 ± 0.33
	2 μM	1.58 ± 0.26	1.58 ± 0.25	1.68 ± 0.25	1.78 ± 0.20	1.90 ± 0.22	2.64 ± 0.25	3.79 ± 0.25
	5 μM	1.58 ± 0.31	1.58 ± 0.29	1.58 ± 0.25	1.60 ± 0.29	1.64 ± 0.26	2.02 ± 0.36	2.77 ± 0.28
	10 μM	1.58 ± 0.25	1.58 ± 0.21	1.58 ± 0.21	1.58 ± 0.21	1.60 ± 0.21	1.65 ± 0.21	1.80 ± 0.21
Shoot length (cm)	Control	7.38 ± 0.37	7.65 ± 0.38	8.20 ± 0.37	8.57 ± 0.38	8.70 ± 0.41	8.90 ± 0.42	9.20 ± 0.41
	2 μM	7.38 ± 0.25	7.38 ± 0.39	7.40 ± 0.33	7.55 ± 0.31	7.62 ± 0.28	8.50 ± 0.35	8.99 ± 0.23
	5 μM	7.38 ± 0.24	7.38 ± 0.26	7.38 ± 0.13	7.42 ± 0.16	7.56 ± 0.12	7.70 ± 0.10	8.38 ± 0.09
	10 μM	7.38 ± 0.37	7.38 ± 0.30	7.38 ± 0.30	7.39 ± 0.29	7.40 ± 0.27	7.60 ± 0.30	8.28 ± 0.32
Secondary root length (cm)	Control	a	0.04 ± 0.00	0.40 ± 0.02	1.01 ± 0.05	1.97 ± 0.22	2.48 ± 0.31	3.54 ± 0.37
	2 μM	a	a	a	a	0.02 ± 0.00	0.02 ± 0.00	0.04 ± 0.02
	5 μM	a	a	a	a	a	0.02 ± 0.00	0.03 ± 0.00
	10 μM	a	a	a	a	a	a	0.02 ± 0.00

a-absent

Fig. 2 Effect of Mercury on root length in *Bacopa monnieri* during growth.

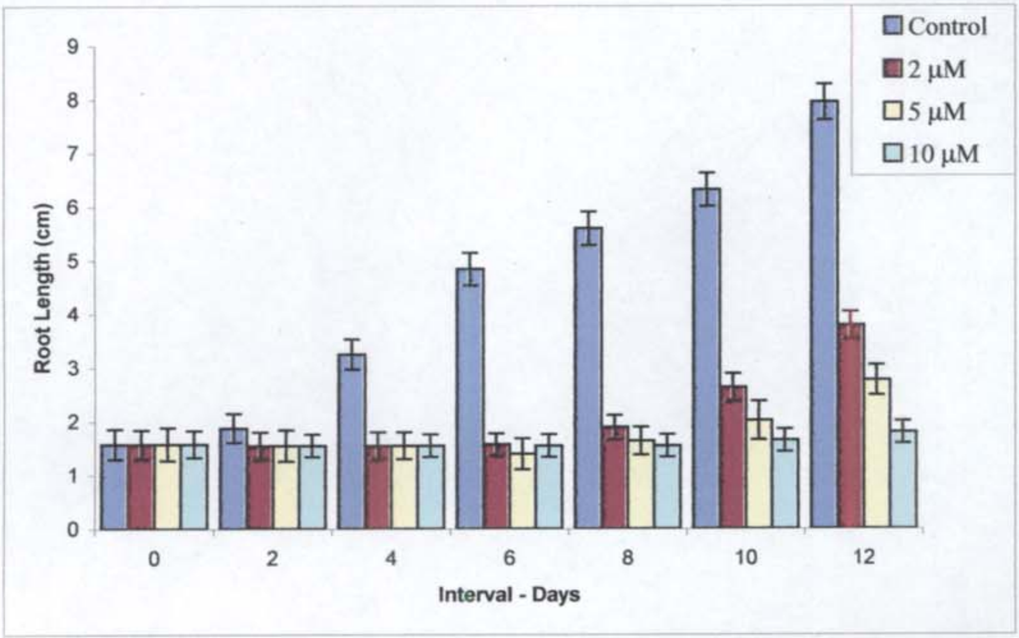
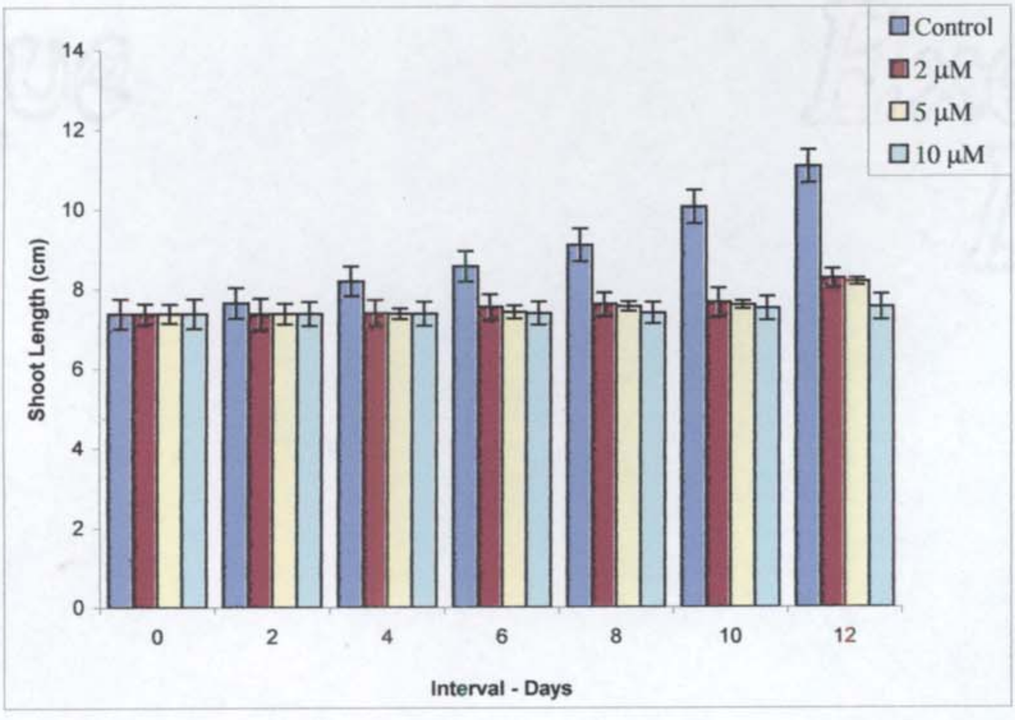


Fig. 3 Effect of Mercury on shoot length in *Bacopa monnieri* during growth.



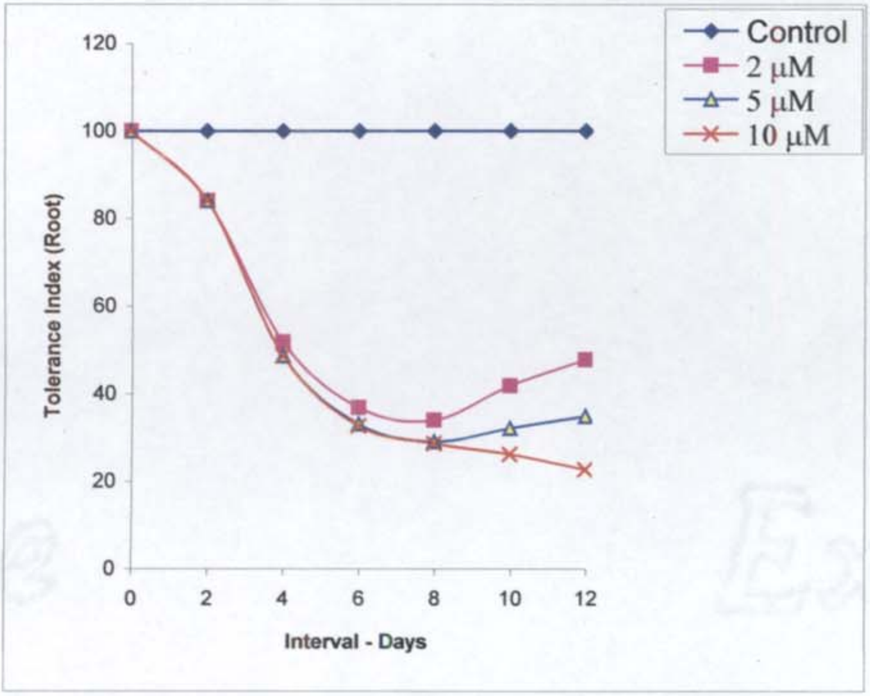
1.4 Tolerance index percentage based on root length

Tolerance index percentage calculated on the basis of root length of *Bacopa monnieri* showed gradual and significant reduction up to 8th day ($P<0.02$) of growth in treatments with 2 and 5 µM concentrations of $HgCl_2$ (Table 2, Fig.4). Afterwards significant increase ($P<0.01$) was shown by plants treated with 2 µM $HgCl_2$ whereas only negligible increase was noticed in plants treated with 5 µM $HgCl_2$. Tolerance index percentage of plants grown at 10 µM concentration was markedly reduced on 4th day ($P<0.02$) and thereafter gradual and continuous reduction ($P<0.01$) was observed (Table 2, Fig.4).

Table: 2 Effect of Mercury on root tolerance index in *Bacopa monneiri* during growth.

Tissue	Treatment concentrations ($HgCl_2$)	Interval - Days						
		0	2	4	6	8	10	12
Root	Control	100	100	100	100	100	100	100
	2 µM	100	84.04 ± 0.33	51.69 ± 0.36	36.77 ± 0.33	33.92 ± 0.44	41.72 ± 0.34	47.67 ± 0.31
	5 µM	100	84.04 ± 0.37	48.61 ± 0.36	33.05 ± 0.38	29.02 ± 0.41	32.06 ± 0.41	34.84 ± 0.28
	10 µM	100	84.04 ± 0.14	48.61 ± 0.29	32.64 ± 0.30	28.57 ± 0.38	26.10 ± 0.44	22.64 ± 0.39

Fig. 4 Effect of Mercury on root tolerance index percentage in *Bacopa monneiri* during growth.



1.5 Stomatal Index

Upper and lower epidermis of *Bacopa monnieri* leaves showed the presence of stomata, more or less uniformly. Treatment with 2 μM $HgCl_2$ showed only negligible variations in stomatal index in both upper and lower epidermis. But due to the treatment of 5 μM $HgCl_2$ the values of stomatal index of upper epidermis were reduced up to 6th day and thereafter increased insignificantly ($P<0.05$). These values were higher in upper epidermis than the lower epidermis during 10–12 days (Table 3). In the lower epidermis, treatment with 5 μM $HgCl_2$ resulted in significant increase ($P<0.02$) compared to the control throughout the experimental period. Similarly 10 μM $HgCl_2$ also showed enhanced values of stomatal index in upper and lower epidermis compared to their respective controls as well as that of the treatments with 2 μM and 5 μM $HgCl_2$. The upper epidermis showed more stomatal index values in comparison with that of the lower epidermis during 8th day onwards (Table 3).

Table: 3 Effect of Mercury on stomatal index in *Bacopa monnieri* during growth.

Interval-Days	Control		Treatment concentrations ($HgCl_2$)					
	Upper epidermis	Lower epidermis	Upper epidermis			Lower epidermis		
	-	-	2 μM	5 μM	10μM	2 μM	5 μM	10μM
0	22.01 ± 1.46	20.00 ± 1.06	21.41 ± 1.98	22.61 ± 1.04	21.25 ± 1.46	23.00 ± 1.02	24.12 ± 1.10	26.12 ± 1.05
2	22.04 ± 0.04	20.98 ± 1.04	21.01 ± 1.46	20.00 ± 1.33	20.98 ± 1.77	23.02 ± 1.33	24.01 ± 1.2	25.03 ± 1.02
4	22.12 ± 0.05	21.68 ± 0.98	21.06 ± 1.74	19.76 ± 1.20	20.93 ± 1.66	24.03 ± 0.96	25.12 ± 1.2	26.10 ± 2.00
6	22.96 ± 0.16	22.73 ± 0.16	22.01 ± 1.28	20.43 ± 1.02	24.48 ± 1.54	23.00 ± 1.34	26.31 ± 1.1	28.11 ± 2.01
8	22.81 ± 0.81	20.65 ± 1.12	22.15 ± 1.50	26.12 ± 1.10	30.00 ± 1.62	24.02 ± 1.2	26.21 ± 1.1	26.30 ± 1.06
10	22.61 ± 1.00	21.42 ± 1.00	23.50 ± 1.44	29.26 ± 1.04	32.82 ± 1.71	25.01 ± 1.21	27.23 ± 1.3	27.31 ± 1.09
12	23.48 ± 1.10	21.95 ± 0.96	23.70 ± 1.30	35.50 ± 1.31	38.00 ± 1.73	23.03 ± 0.96	27.15 ± 1.4	29.23 ± 1.20

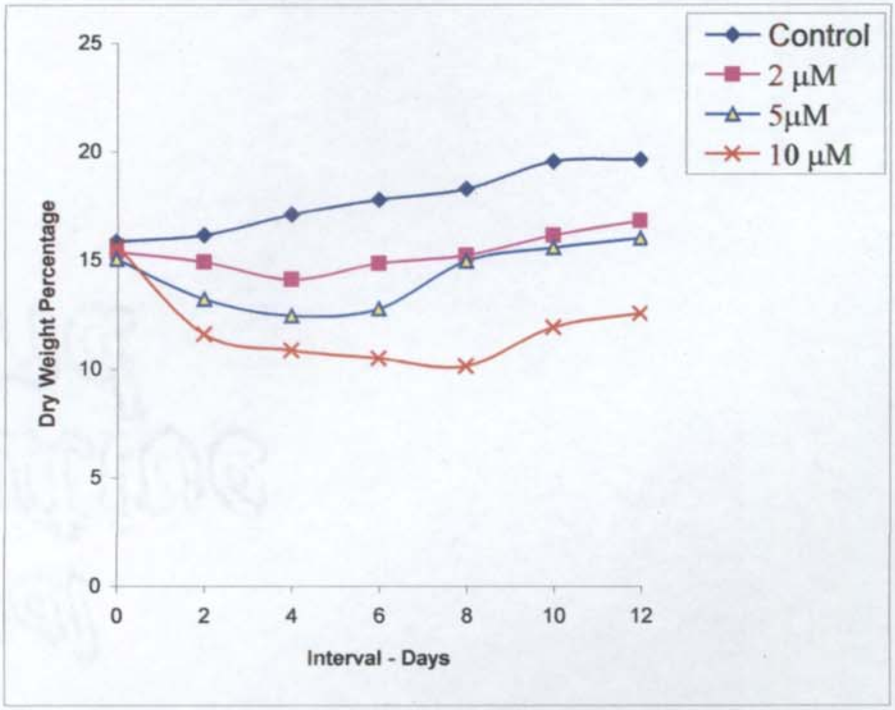
1.6 Dry weight percentage distribution

Dry matter content of *Bacopa monnieri* showed reduction in all treatments at all intervals compared to the control (Table 4, Fig.5). During the growth, dry matter was reduced gradually but insignificantly ($P<0.05$) and only negligible increase was shown after 8 days in propagules treated with 2 μM $HgCl_2$ solution ($P<0.02$). Almost the same trend was shown by plants treated with 5 μM concentration which exhibited slight increase after 8 days of treatment. Plants treated with 10 μM $HgCl_2$ exhibited a significant reduction of dry weight on 2nd day and thereafter only negligible reduction was observed up to 8th day followed by slight increase on 10th and 12th days. When a comparison is made between treatments, minimum dry matter was present in 10 μM treatment.

Table: 4 Effect of Mercury on dry weight percentage distribution in *Bacopa monnieri* during growth.

Interval- Days	Control	Treatment concentrations ($HgCl_2$)		
		2 μM	5 μM	10 μM
0	15.85 ± 0.42	15.36 ± 0.52	15.04 ± 0.55	15.65 ± 0.54
2	16.15 ± 0.39	14.90 ± 0.54	13.20 ± 0.61	11.59 ± 0.49
4	17.09 ± 0.51	14.11 ± 0.61	12.43 ± 0.62	10.84 ± 0.62
6	17.77 ± 0.34	14.84 ± 0.67	12.75 ± 0.67	10.47 ± 0.56
8	18.27 ± 0.24	15.20 ± 0.53	14.93 ± 0.52	10.11 ± 0.57
10	19.53 ± 0.18	16.12 ± 0.52	15.55 ± 0.63	11.89 ± 0.52
12	19.61 ± 0.36	16.79 ± 0.59	16.00 ± 0.71	12.53 ± 0.61

Fig.5 Effect of Mercury on dry weight percentage distribution in *Bacopa monnieri* during growth.



2 ANATOMICAL/HISTOCHEMICAL STUDIES

2.1 Root

Microtome sections of roots treated with 2, 5 and 10 μM concentrations of $HgCl_2$, stained with safranin showed thick and distorted piliferous layer in which dark deposits are seen. Stained masses are also seen in the xylem vessels of root. Longitudinal sections of roots showed translocation of Mercury as elongated patches/columns through the undifferentiated vascular tissues (Fig. 6a, A-D2).

2.2 Stem

Free-hand sections of stem stained with safranin showed typical stem anatomy of aquatic plants (Fig. 6b, A&B). Cross section of stem of control plants consisted of compactly arranged single layered epidermis and broad cortex comprising parenchymatous (aerenchyma) cells with plenty of air spaces (Fig. 6b, A&B). The stele consisted of number of vascular bundles with uniseriate xylem vessels and conspicuous phloem constituting small cells. Endodermis comprised of single layered compactly arranged cells. Conspicuous pith consisted of large parenchymatous cells without any intercellular spaces (Fig. 6b, A&B).

Stem cross sections of propagules treated with 2 μM $HgCl_2$ did not show any significant anatomical change in the epidermis and cortex compared to the control, but endodermis cells became conspicuous with thick walled cells. Phloem cells showed slight shrinkage and xylem contained dark coloured deposits filling almost all metaxylem vessels in general and protoxylem in particular. Such stained masses were absent in the pith, endodermis and cortical cells (Fig. 6b, C&D). Stem cross sections of control plants showed no stained inclusions so the lumens of the vessels were empty (Fig.6b, A&B).

Plants treated with 5 μM $HgCl_2$ also showed almost similar pattern of accumulation of coloured (stained) masses filling the lumen of the vessels (Fig. 6b, E&F).

Cross sections of stem of plants treated with 10 μM $HgCl_2$ showed slight degradation of cortical cells, where endodermis and phloem cells were similar to other treatments. Maximum stained masses filling all metaxylem and protoxylem vessels were present in these sections. Localisation of stained masses were absent in the pith, endodermis and cortex in all treatments (Fig. 6c, G&H).

Fig. 6a

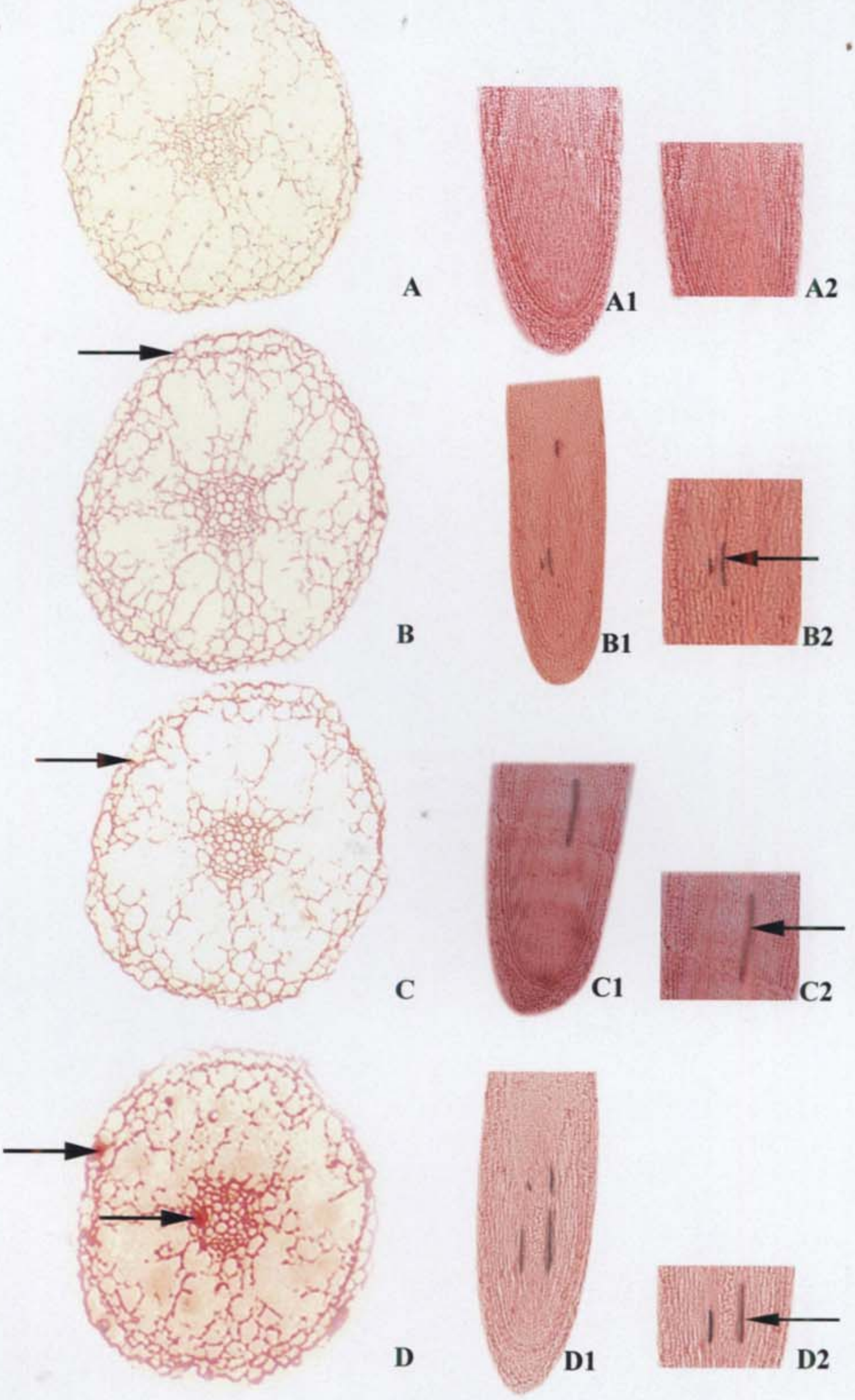


Fig. 6a - *Bacopa monnieri* treated with $HgCl_2$
A-D2 (Root)

A - Control (C.S)
A1- Control (L.S)
A2- Enlarged

B - 2 μM (C.S)
B1- 2 μM (L.S)
B2- Enlarged

C - 5 μM (C.S)
C1- 5 μM (L.S)
C2- Enlarged

D - 10 μM (C.S)
D1- 10 μM (L.S)
D2- Enalrged

Arrow denotes thick distorted cell walls/ Hg deposits

Fig. 6b

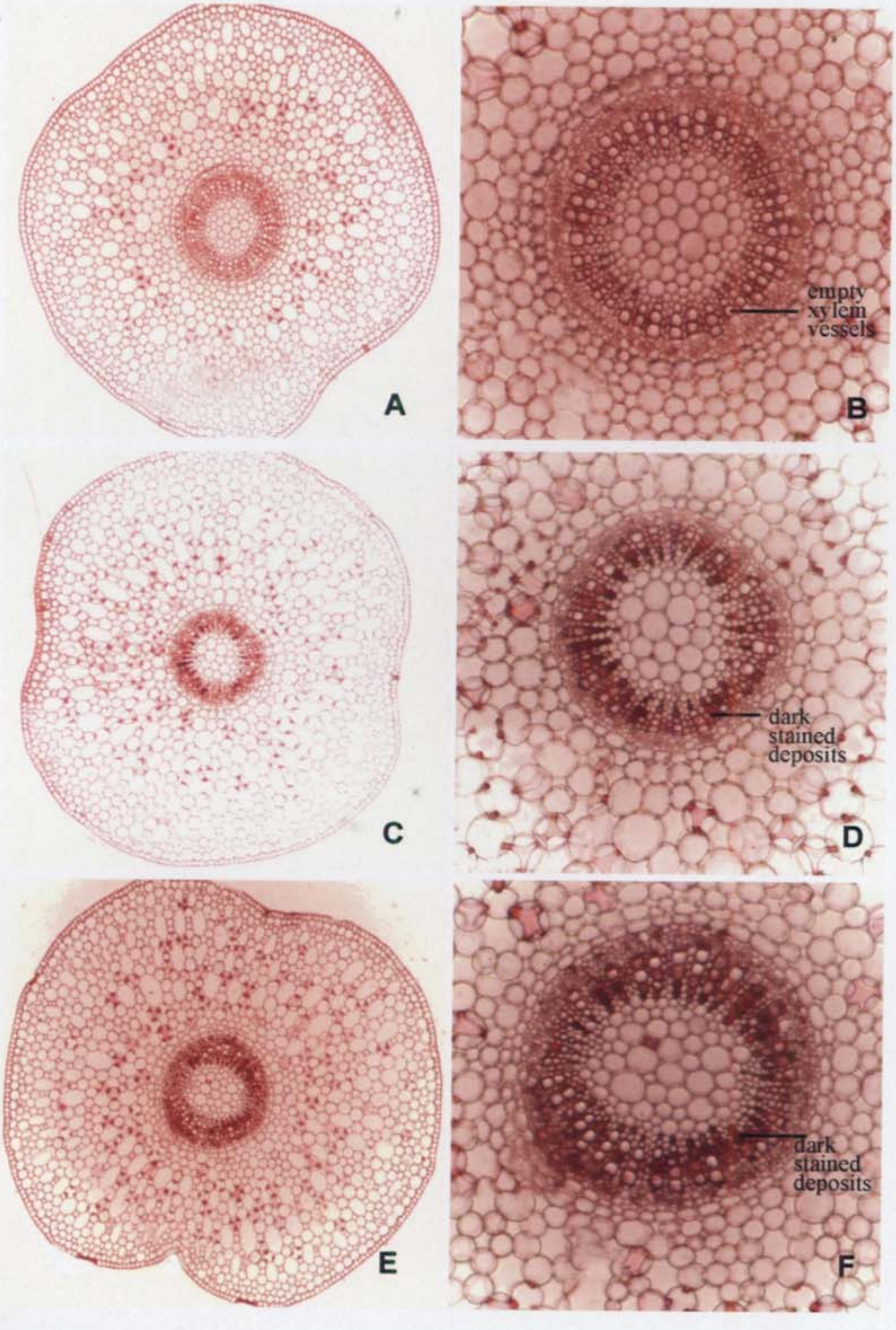


Fig. 6b A-F Stem cross sections - *Bacopa monnieri* treated with $HgCl_2$ (Stained with safranin)

A- Control -ground plan(Hoagland solution)	D- Enlarged stele
B - Enlarged stele	E - 5μM
C - 2 μM	F - Enlarged stele

Fig. 6c

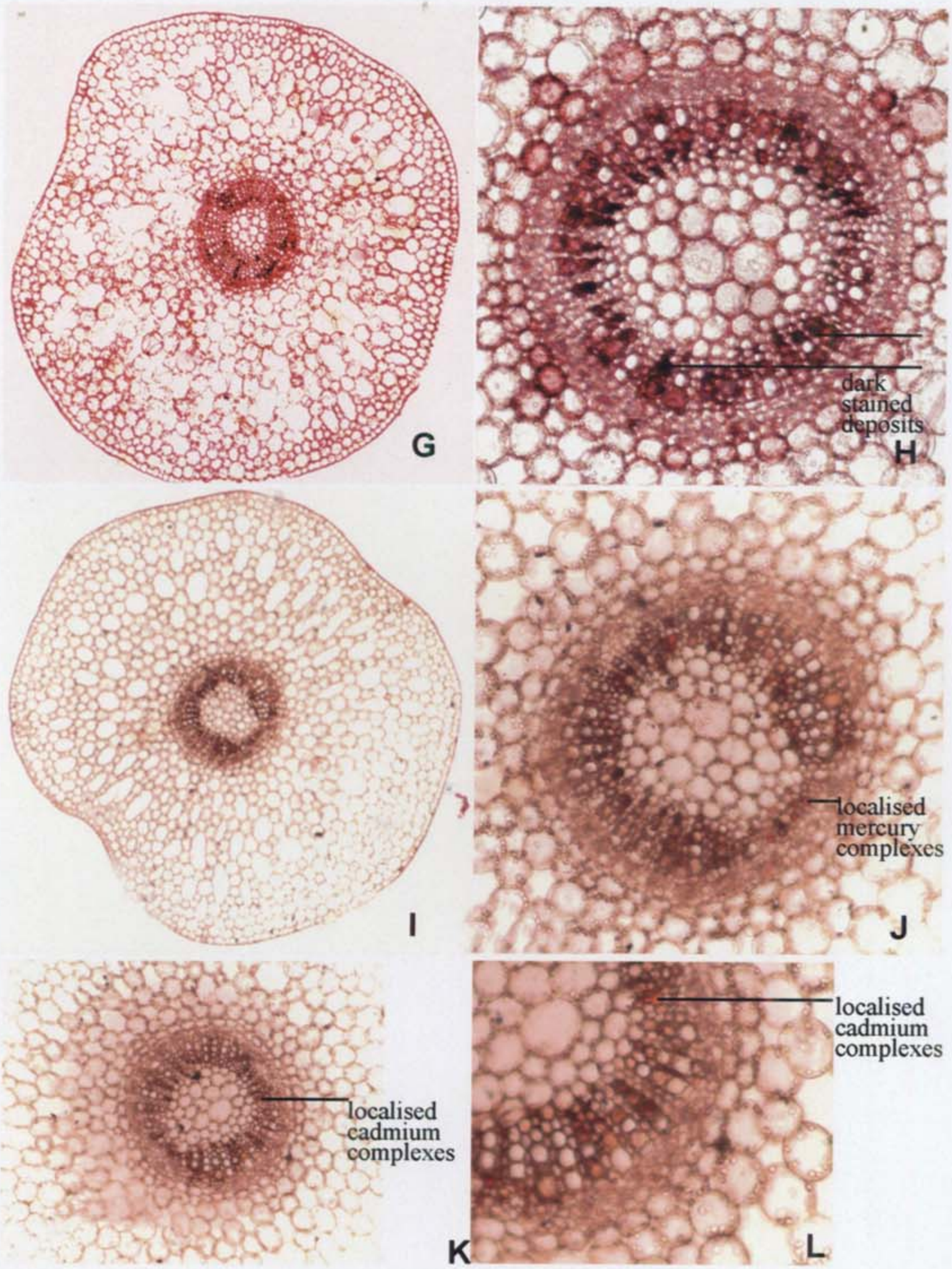


Fig. 6c G-L Stem cross sections - *Bacopa monnieri* treated with $HgCl_2$

G- 10 µM
H - Enlarged stele

I- 10 µM (dithizone stained)
J - Enlarged stele
K- 30 µM Cd treated
(dithizone stained)
L - Stele (one portion enlarged)

Fig. 6d

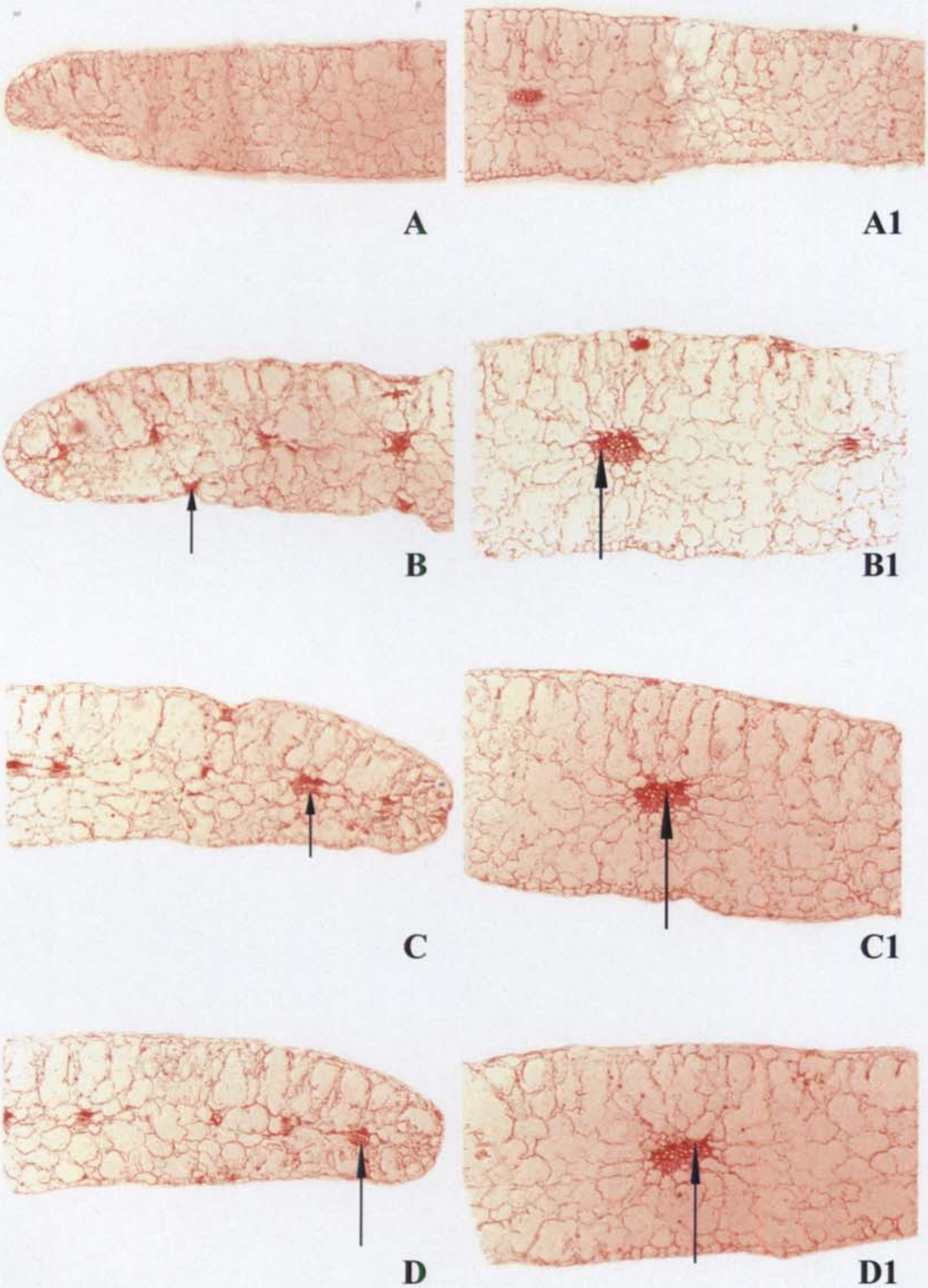


Fig. 6d *Bacopa monnieri* treated with $HgCl_2$
A-D1 (Leaf C.S)

A&A1- Control B&B1- 2 µM C&C1- 5 µM D &D1- 10 µM

Arrow denotes stained deposits of Hg

Sections stained with dithizone also showed orange coloured stained masses in the xylem vessels of the tissues treated with 10 µM $HgCl_2$ and 30 µM $CdCl_2$ (Fig. 6c, I,J & K,L).

2.3 Leaf

The cross sections of leaves treated with 2, 5 and 10 µM concentrations of $HgCl_2$ showed some stained patches near the leaf margins and surrounding the vascular bundles when stained with safranin, which are absent in control leaf (Fig. 6d, A-D1).

3. PHYSIOLOGICAL AND BIOCHEMICAL STUDIES

3.1. Total protein

Protein content of *Bacopa monnieri* propagules treated with 2 and 5 µM $HgCl_2$ showed significant reduction ($P<0.02$) from 2nd day to 4th day compared to the control (Table 5, Fig. 7). Thereafter the protein content was significantly increased in 2 and 5 µM treatments during 6th and 8th days ($P<0.02$ and 0.02 respectively). After 8th day slight increase was observed in both of these treatments. Sharp decline of protein content was shown by propagules treated with 10 µM during 2-6 days of growth and again gradually increased on 8th day. Significant increase in protein content was observed during 8-10 days ($P<0.02$ and $P<0.01$ respectively). The quantity of protein content during the period of 10-12 days of 10 µM $HgCl_2$ treatment was almost equal to that of the control (Table 5, Fig. 7).

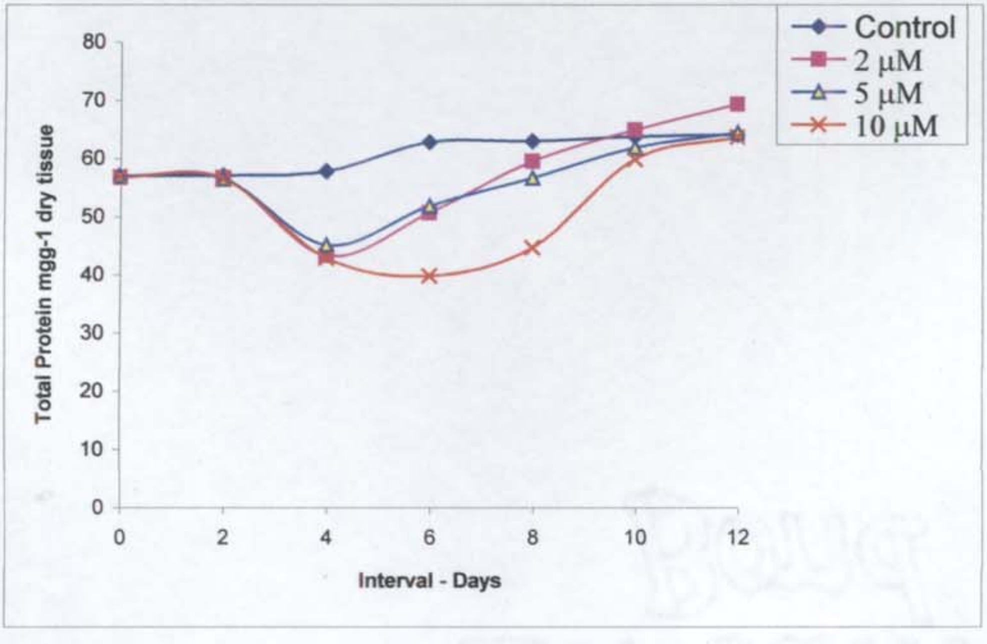
Table: 5 Effect of Mercury on total protein content in *Bacopa monnieri* during growth.

(mg g^{-1} dry tissue)

Treatment concentrations ($HgCl_2$)	Interval - Days						
	0	**2**	**4**	**6**	**8**	**10**	**12**
Control	56.98 ±0.49	57.08 ± 0.39	57.79 ±0.61	62.79 ± 0.34	62.96 ±0.24	63.74 ± 0.18	64.08 ± 0.36
2 µM	56.75 ± 0.55	56.68 ± 0.54	43.29 ±0.62	50.64 ±0.67	59.44 ±0.53	64.84 ± 0.52	69.33 ± 0.59
5 µM	56.99 ±0.74	56.47 ±0.61	45.16 ±0.62	51.84 ± 0.56	56.64 ±0.52	61.83 ± 0.63	64.42 ±0.71
10 µM	56.86 ± 0.80	56.49 ±0.49	42.88 ±0.47	43.79 ±0.56	44.64 ±0.51	59.84 ±0.52	63.68 ±0.61

Fig. 7 Effect of Mercury on total protein content in *Bacopa monnieri* during growth.

(mg g^{-1} dry tissue)



3.2. Protein profile (PAGE)

Electrophoretic pattern of protein profile showed more number of very thin bands of molecular weight between 47.2 and 79.8 kDa in plants treated with 5 and 10 μM $HgCl_2$ on 2nd day in comparison with that of the control (Fig. 8). But on 4thday number of protein bands in the plants treated with 5 and 10 μM $HgCl_2$ were increased compared to that of the 2nd day. No significant difference was observed in the protein profile of plants treated with $HgCl_2$ on 6th, 8th and 10th days generally. Similarly, difference between protein profile of 5 μM and 10 μM treatments was not evident. But 12th day samples showed narrow but distinct bands of molecular weight between 50.4 and 79.8 kDa which were present in the 4th and 8th day intervals. The narrowing of these bands were gradual towards 12th day (Fig. 8). However, more low molecular weight thin protein bands were appeared in the protein profile of 10th and 12th day samples (Fig. 8).

3.3. Chlorophylls

Plant leaves treated with 2 μM $HgCl_2$ showed an insignificant increase ($P<0.05$) in chlorophyll-a content on 2nd day and remained unchanged up to 8th day but again slight increase was observed afterwards ($P<0.02$ and $P<0.05$ respectively) compared to the control (Table 6, Fig. 9).

Chlorophyll-b pigment also showed slight increase ($P<0.05$) on 2nd day and remained unchanged throughout the experimental period. A significant increase in total chlorophyll was observed on 2nd day and remained unchanged up to 8th day followed by slight enhancement in total chlorophyll. Chl-a/b ratio was gradually increasing up to 4th day and remained unchanged throughout the experimental period. In the control

Fig.8

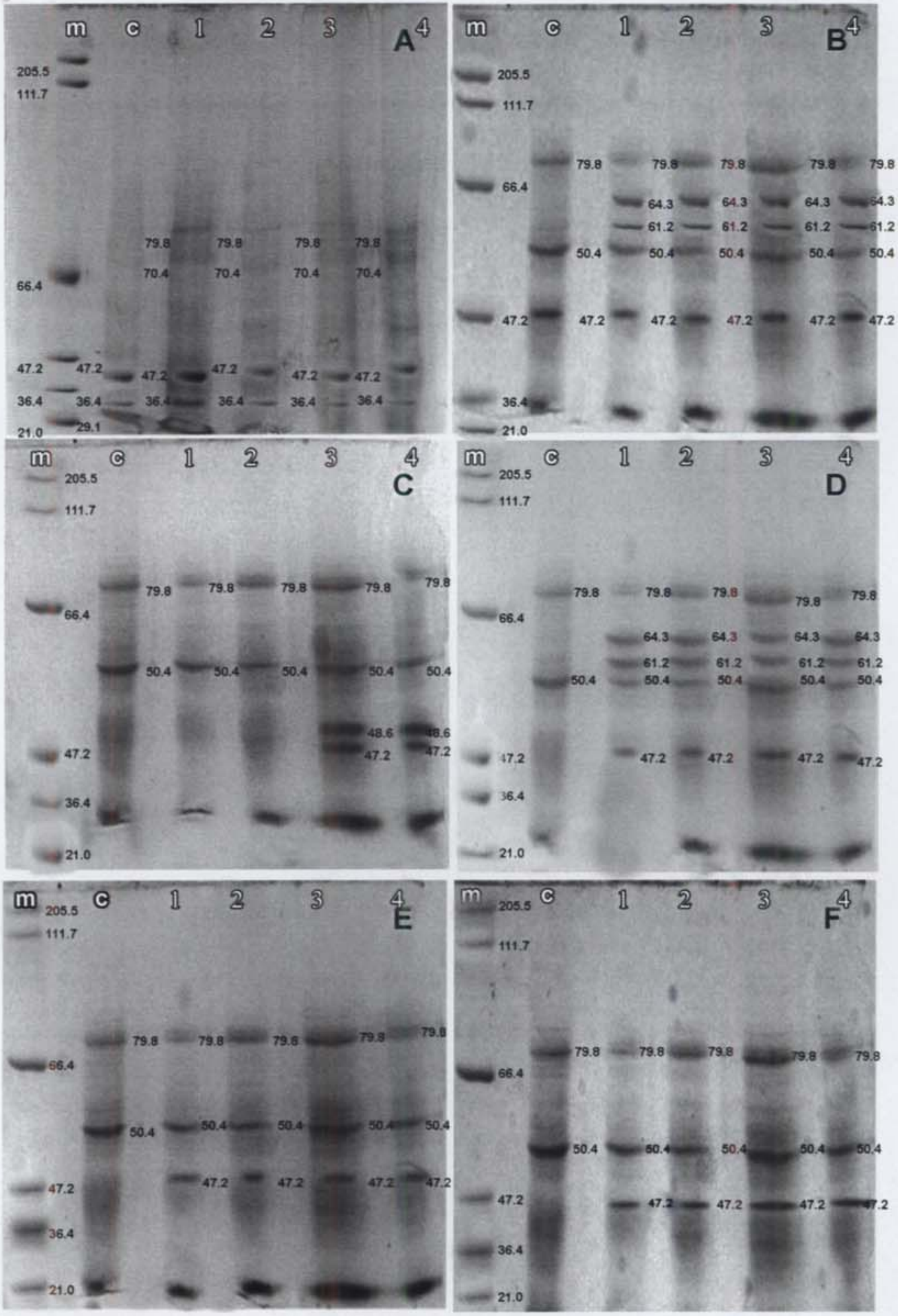


Fig. 8 Protein profile of *Bacopa monnieri* treated with $HgCl_2$ and $CdCl_2$ sampled at different intervals.

m- marker c - control 1 - 5µM Hg 2 - 10µM Hg 3 - 20µM Cd 4 - 30µM Cd

A- 2 days B- 4 days C- 6 days D- 8 days E- 10 days F- 12 days

a/b ratio was gradually (insignificant) increasing throughout the growth *i.e.,* 12 days.

In propagules treated with 5 μM $HgCl_2$ Chl-a, b, total and a/b ratio remained unchanged up to 4th day and thereafter significant increase was observed in chl-a, total and a/b ratio. During 10–12 days, Chl-a, total and a/b ratio showed significant ($P<0.02$) increase. Chlorophyll-b exhibited only insignificant ($P<0.05$) but gradual increase throughout the experimental period.

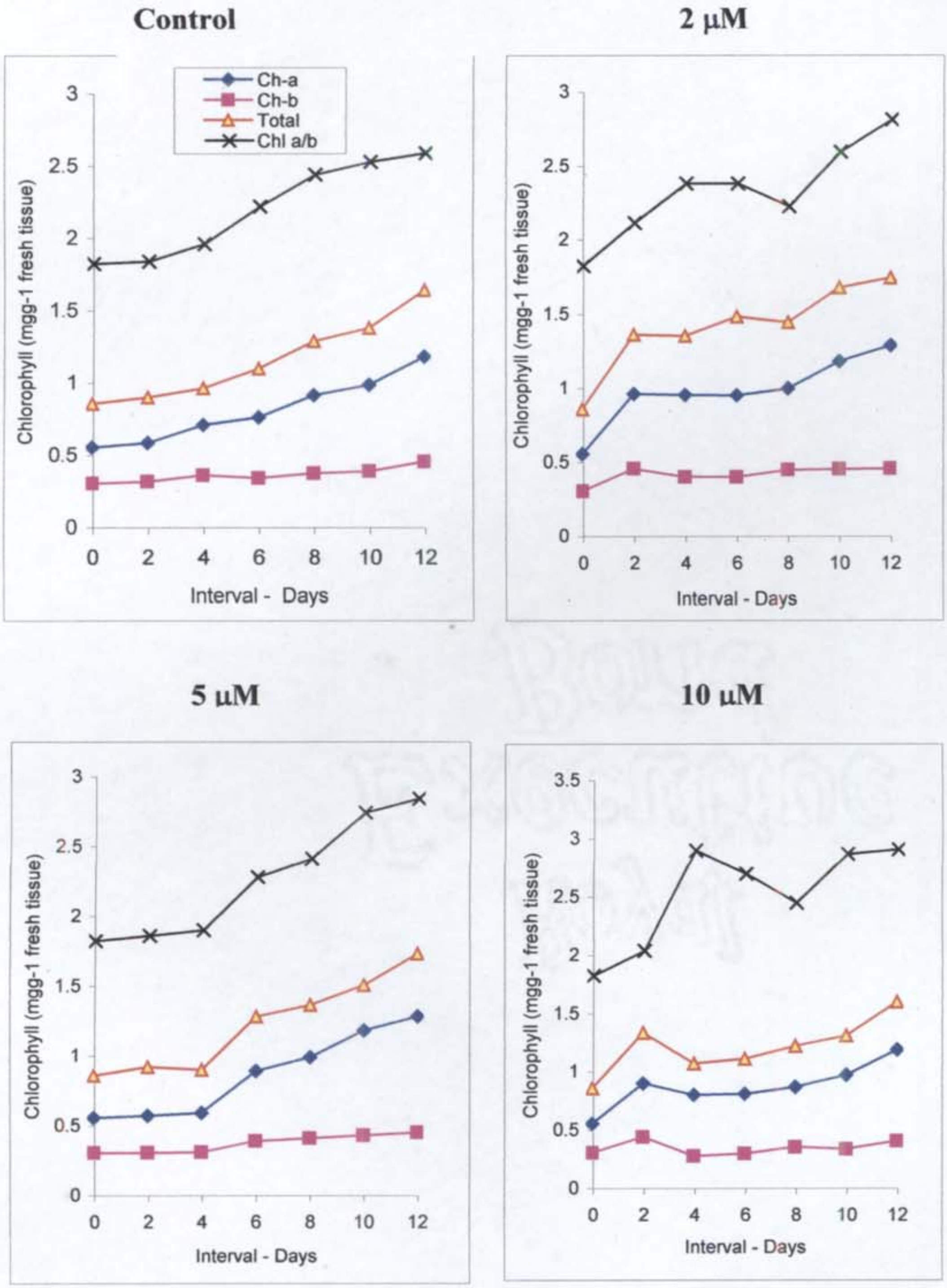
Plants treated with 10 μM $HgCl_2$ showed a highly significant ($P<0.01$) increase of Chl-a, b, total and a/b ratio on 2nd day in comparison with that of the control (Table 6, Fig. 9). But insignificant reduction of Chl-b and total chlorophyll ($P<0.05$ and $P<0.05$ respectively) was observed on 4th day where as Chl-a showed only slight reduction resultantly a/b ratio was remarkably increased. Almost same pattern was followed on 6th and 8th days with slight reduction in a/b ratio. After this interval there occurred slight increase in Chl-a, b and total chlorophyll and with a significant increase in a/b ratio. In samples of 12th day, insignificant increase was observed in Chl-a, b, total and a/b ratio.

Table: 6 Effect of Mercury on chlorophyll content in *Bacopa monnieri* during growth.
(mg g^{-1} fresh tissue)

Treatment concentrations ($HgCl_2$)	Chlorophylls	Interval - Days						
		0	2	4	6	8	10	12
Control	Chl-a	0.55±0.01	0.58±0.01	0.71±0.01	0.76±0.02	0.91±0.03	0.98±0.04	1.18±0.04
	Chl-b	0.30±0.01	0.31±0.01	0.36±0.01	0.34±0.01	0.37±0.01	0.39±0.01	0.45±0.01
	Total	0.85±0.02	0.90±0.02	0.96±0.03	1.10±0.04	1.29±0.05	1.38±0.05	1.64±0.06
	Chl a/b	1.82±0.03	1.84±0.04	1.96±0.06	2.22±0.07	2.44±0.08	2.53±0.09	2.59±0.10
2 μM	Chl-a	0.55±0.02	0.96±0.03	0.95±0.03	0.95±0.03	0.99±0.04	1.18±0.04	1.28±0.05
	Chl-b	0.30±0.01	0.45±0.01	0.40±0.01	0.39±0.01	0.44±0.01	0.45±0.01	0.45±0.01
	Total	0.85±0.02	1.36±0.02	1.35±0.03	1.48±0.04	1.44±0.04	1.68±0.05	1.74±0.05
	Chl-a/b	1.82±0.03	2.11±0.04	2.38±0.04	2.38±0.04	2.22±0.03	2.59±0.04	2.81±0.04
5 μM	Chl-a	0.55±0.01	0.57±0.01	0.59±0.02	0.89±0.02	0.99±0.02	1.18±0.03	1.28±0.04
	Chl-b	0.30±0.01	0.30±0.01	0.31±0.01	0.39±0.02	0.41±0.02	0.43±0.02	0.45±0.02
	Total	0.85±0.02	0.92±0.02	0.90±0.02	1.28±0.03	1.36±0.03	1.50±0.04	1.73±0.05
	Chl-a/b	1.82±0.03	1.86±0.03	1.90±0.04	2.28±0.04	2.41±0.04	2.74±0.05	2.84±0.05
10 μM	Chl-a	0.55±0.02	0.89±0.02	0.80±0.02	0.81±0.02	0.87±0.02	0.97±0.03	1.19±0.04
	Chl-b	0.30±0.01	0.44±0.02	0.27±0.01	0.29±0.01	0.35±0.01	0.33±0.01	0.40±0.01
	Total	0.85±0.02	1.33±0.02	1.07±0.02	1.11±0.03	1.22±0.03	1.31±0.03	1.60±0.04
	Chl-a/b	1.82±0.03	2.04±0.04	2.90±0.04	2.70±0.04	2.45±0.04	2.87±0.05	2.90±0.06

Fig. 9 Effect of Mercury on chlorophyll content in *Bacopa monnieri* during growth.

(mg g^{-1} fresh tissue)



Control

2 µM

5 µM

10 µM

4. BIOACCUMULATION STUDIES

4.1. Bioaccumulation of Mercury (during 12 days treatment)

During growth for 12 days, accumulation of Mercury was maximum in roots followed by stem and leaves. The accumulation of Hg in stem and leaves was increasing proportional to the concentration of the metal and period of growth. But roots showed only negligible changes (Table 7a, Fig.10). Mercury accumulated in the root tissue was almost doubled, compared to the stem in all treatments of both concentrations. Leaf tissue showed gradual, but enhanced rate of accumulation than the other tissue during the period of 12 days. When a comparison is made between 5 μM and 10 μM concentrations Hg accumulated was not proportional to the concentration. Only marginal increase was observed in the tissues treated with 10 μM compared to 5 μM $HgCl_2$. However, leaf tissue of 10 μM treatment showed more enhancements in the accumulation of Hg compared to 5 μM during 12 days growth.

For bioaccumulation studies of Hg and Cd, *Bacopa monnieri* plants were grown in Hoagland solution containing a known quantity of these metals. Hence, a comparison between accumulation (Content/tissue cultivated) and quantity of metal retained (residual) in the medium during 12 days of growth was calculated in order to ascertain the patterns of distribution of these elements (Table 7b). Mercury given in the two concentrations showed only marginal increase between the contents of accumulation in plants at each interval, whereas Hg content left behind during the intervals showed gradual reduction and almost exhausted on 12th day. When the loss of Hg calculated as the difference between amount given and the sum of accumulation and residual showed

Table: 14a Accumulation of Cadmium contents in different parts of *Bacopa monnieri* treated with $CdCl_2$ during growth.
mg g^{-1} dry tissue (concentration)

Concentrations		Plant parts	Interval-Days					
			2	4	6	8	10	12
$CdCl_2$	20 µM	Root	9.1± 0.03	120.1± 3.2	164.1± 3.5	199.0± 3.5	200.1± 3.6	200.9± 3.6
		Stem	4.0± 0.02	8.0± 0.03	34.0± 1.8	87.9± 2.5	105.8± 3.0	106.1± 3.0
		Leaf	NDR	2.0± 0.01	3.0± 0.01	4.0± 0.02	6.0± 0.02	8.0± 0.03
	30 µM	Root	12.7± 0.05	131.0± 3.2	230.0± 4.1	242.0± 4.2	238.0± 4.2	256.0± 4.4
		Stem	8.6± 0.04	8.0± 0.04	54.0± 2.5	100.0± 3.0	135.0± 3.3	187.0± 3.6
		Leaf	4.0± 0.01	6.0± 0.02	10.0± 0.04	13.0± 0.05	13.0± 0.05	15.0± 0.06

NDR- Non Detectable Range

Fig. 10 Accumulation of Mercury contents in different parts of *Bacopa monnieri* treated with $HgCl_2$ during growth.
$\mu g\ g^{-1}$ drytissue(concentration)

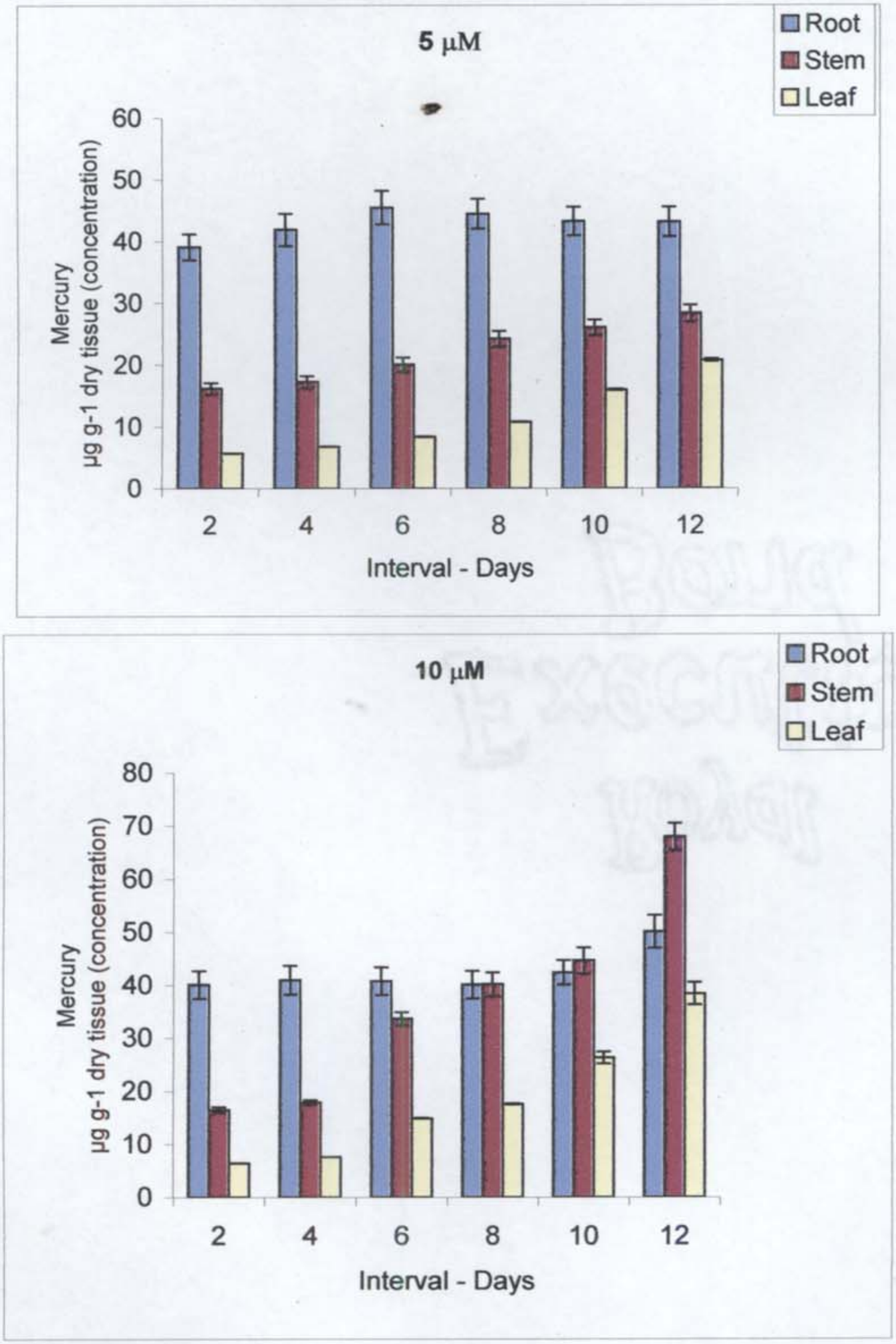


Table: 7b Percentage distribution of Mercury in *Bacopa monnieri* in relation to the availability and loss during growth.

µg/whole plants (Content)

Treatment	Quantity given		Interval-Days					
			2	4	6	8	10	12
$HgCl_2$	5 µM (200µg Hg)	A	61 (30.5)	66 (32.2)	74 (37.0)	79 (39.5)	85 (42.5)	92 (46.0)
		R	58 (29.0)	42 (21.0)	30 (15.0)	22 (11.0)	13 (6.5)	4 (2.0)
		L	81 (40.5)	93 (46.5)	96 (48.0)	99 (49.5)	102 (51.0)	104 (52.0)
	10 µM (400µg Hg)	A	63 (15.5)	66 (16.5)	89 (23.2)	98 (24.5)	113 (28.0)	156 (39.0)
		R	205 (51.2)	190 (47.5)	146 (36.5)	118 (29.5)	81 (20.2)	5 (1.3)
		L	133 (33.2)	144 (36.0)	161 (40.2)	184 (46.0)	207 (51.7)	239 (59.7)

Values in parenthesis are percentage distributions

A - Total accumulation in plants (µg/whole tissue)
R - Residual content (µg) present in the medium, during 12 days of growth
L - Quantity (µg) lost during 12 days growth (difference between accumulation + residue and total Mercury content given).

4. 2. Bioaccumulation of Mercury (during 50 days treatments)

When plants were exposed to repeated dozes of $HgCl_2$ at an interval of 10 days (20th, 30th, 40th, 50th) during a period of 50 days of growth, Mercury accumulation in the plant was increased proportional to the concentration (Table 7c). But residual amount remained unchanged and slight increase in the loss of Hg was observed. The percentage distribution of accumulation was almost uniform irrespective of the period and concentration.

Ten μM concentrations of $HgCl_2$ during 50 days of growth at an interval of 10 days also showed more accumulation but percentage distribution was lower than that of 5 μM. Proportional increase in residual Hg was shown but percentage did not change. Loss of Hg showed slight increase but the percentage distribution slightly reduced (Table 7c).

Table: 7c Bioaccumulation of Mercury in *Bacopa monnieri* during repeated exposure of $HgCl_2$ up to 50 days

µg/whole plants (content)

Treatment	Concentration		Interval-Days			
			20	30	40	50
			Quantity given			
			250 µg	300 µg	350 µg	400 µg
$HgCl_2$	5 µM	A	98 (39.2)	112 (37.3)	148 (42.2)	174 (43.5)
		R	43 (12.2)	74 (24.6)	76 (21.7)	92 (23.0)
		L	109 (43.6)	114 (38.0)	126 (36.0)	134 (33.5)
			Quantity given			
	10 µM		500 µg	600 µg	700 µg	800 µg
		A	121 (24.2)	164 (27.3)	189 (27.0)	204 (25.5)
		R	160 (32.0)	197 (39.4)	247 (35.2)	290 (36.2)
		L	219 (43.8)	239 (47.8)	264 (37.7)	306 (38.2)

Values in parenthesis are percentage distributions

A - Total accumulation in plants (µg/whole tissue)
R - Residual content (µg) present in the medium, during 50 days growth

L - Quantity (µg) lost during 50 days growth (difference between accumulation + residue and total Mercury content given).

EFFECTS OF $CdCl_2$

1. MORPHOLOGICAL MEASUREMENTS

Bacopa monnieri plants treated with $CdCl_2$ at 10, 20 and 30 μM concentrations also showed morphological variations. Inhibition of root growth resulting in stunted nature of root was observed on 2nd day onwards. Initiation of secondary root was delayed in all concentrations compared to the control (Fig. 1 f,e,d).

1.1. Root length

In comparison with control, slight but gradual elongation of root was occurred in 10 μM, but in 20 μM concentration root growth inhibition up to 6 days and afterwards gradual increase was observed in 30 μM. A general growth inhibition was observed for total experimental period (Table 8, Fig.11a). The increase was inversely proportional to the increasing concentration of Cd. However, significant increase in root length was shown by plants treated with 10 μM concentrations of $CdCl_2$ for 2–12 days ($P<0.01$), whereas in 20 μM treatment, the root length increase was negligible compared to the control. In 30 μM concentration the root growth inhibition was continued almost constantly.

1.2. Shoot length

Plants treated with 10, 20 and 30 μM concentrations of $CdCl_2$ showed inhibited growth of shoot up to 8th day of treatment. In 10 μM treatment showed a slight increase and afterwards shoot length was increased gradually (Table 8, Fig.11b). The increase in shoot length was inversely proportional to the concentration of $CdCl_2$. Significant shoot growth was observed only in the treatment of 10 µM $CdCl_2$ ($P<0.01$) compared to other treatments during 10–12 days.

1.3. Secondary roots

In the case of plants treated with $CdCl_2$, secondary root initiation started on 4th day in 10 µM and on 8th day in 20 µM Cd treatment. Secondary roots were totally absent in 30 µM $CdCl_2$ (Table 8.) treatment.

Table: 8 Effect of Cadmium on the root length, shoot length, and secondary root length in *Bacopa monnieri* during growth.

Tissue	Treatment concentrations ($CdCl_2$)	Interval - Days						
		0	2	4	6	8	10	12
Root length (cm)	Control	1.58 ± 0.28	1.88 ± 0.27	3.25 ± 0.28	4.84 ± 0.30	5.60 ± 0.31	6.32 ± 0.31	7.95 ± 0.33
	10 µM	1.58 ± 0.20	1.58 ± 0.20	1.70 ± 0.20	1.79 ± 0.22	1.97 ± 0.26	2.40 ± 0.22	3.50 ± 0.28
	20 µM	1.58 ± 0.20	1.58 ± 0.18	1.58 ± 0.25	1.58 ± 0.16	1.71 ± 0.16	1.90 ± 0.18	2.50 ± 0.16
	30 µM	1.58 ± 0.37	1.58 ± 0.38	1.58 ± 0.38	1.58 ± 0.40	1.58 ± 0.40	1.58 ± 0.40	1.58 ± 0.40
Shoot length (cm)	Control	7.38 ± 0.37	7.65 ± 0.38	8.20 ± 0.37	8.57 ± 0.38	9.10 ± 0.41	10.05 ±0.42	11.07 ±0.41
	10 µM	7.38 ± 0.36	7.38 ± 0.35	7.38 ± 0.29	7.68 ± 0.43	8.02 ± 0.35	8.55 ± 0.25	9.05 ± 0.23
	20 µM	7.38 ± 0.48	7.38 ± 0.32	7.38 ± 0.43	7.38 ± 0.47	7.67 ± 0.38	8.07 ± 0.48	8.38 ± 0.48
	30 µM	7.38 ± 0.47	7.38 ± 0.41	7.38 ± 0.44	7.38 ± 0.45	7.38 ± 0.40	7.38 ± 0.37	7.38 ± 0.42
Secondary root length(cm)	Control	a	0.02 ± 0.00	0.02 ± 0.00	0.04 ± 0.02	0.98 ± 0.02	1.47 ± 0.03	2.68 ± 0.09
	10 µM	a	a	0.02 ± 0.00	0.02 ± 0.00	0.07 ± 0.00	0.09 ± 0.00	1.01 ± 0.00
	20 µM	a	a	a	a	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
	30 µM	a	a	a	a	a	a	a

a – absent

17

Fig. 11a Effect of Cadmium on root length in *Bacopa monnieri* during growth.

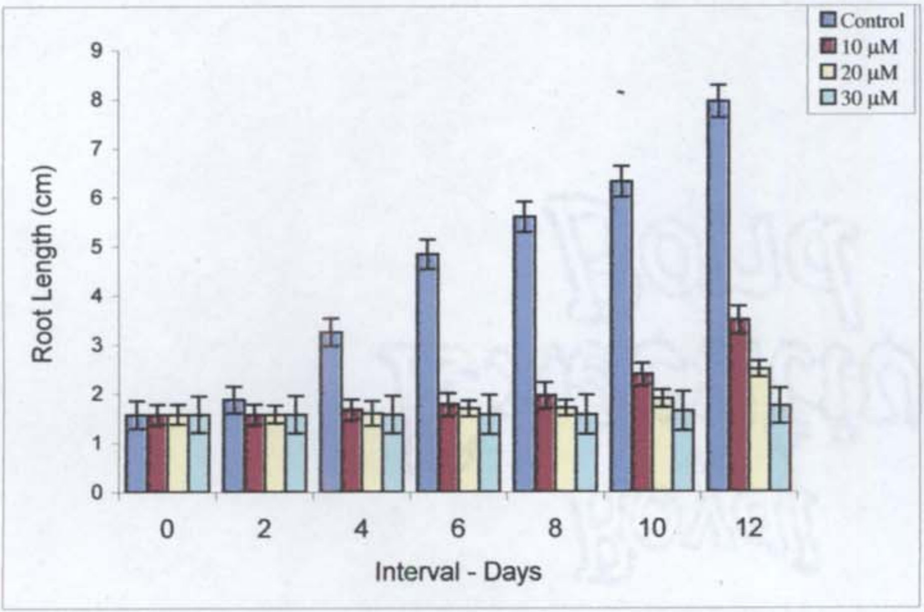
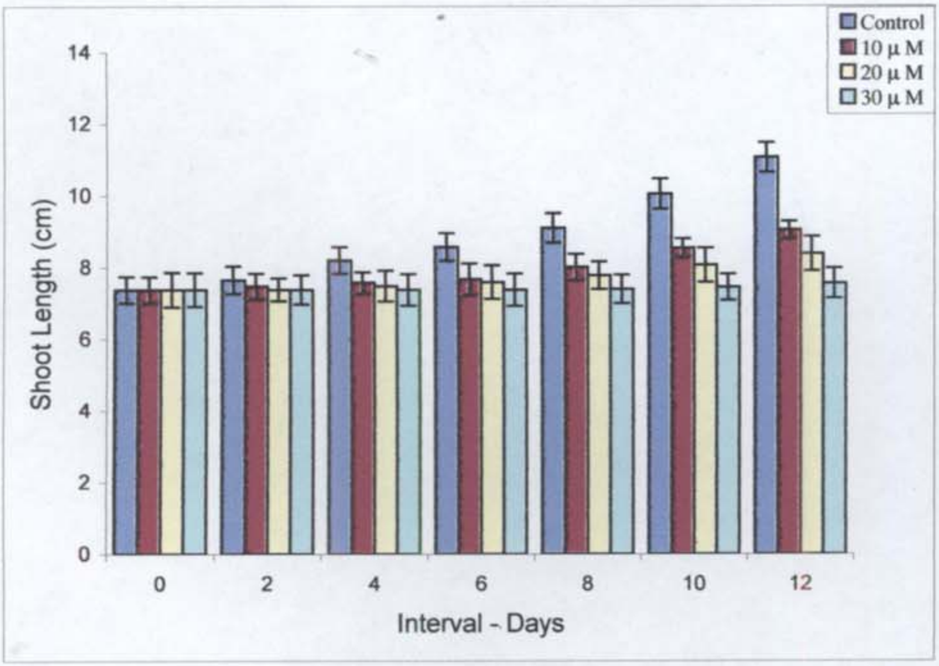


Fig. 11b Effect of Cadmium on shoot length in *Bacopa monnieri* during growth.



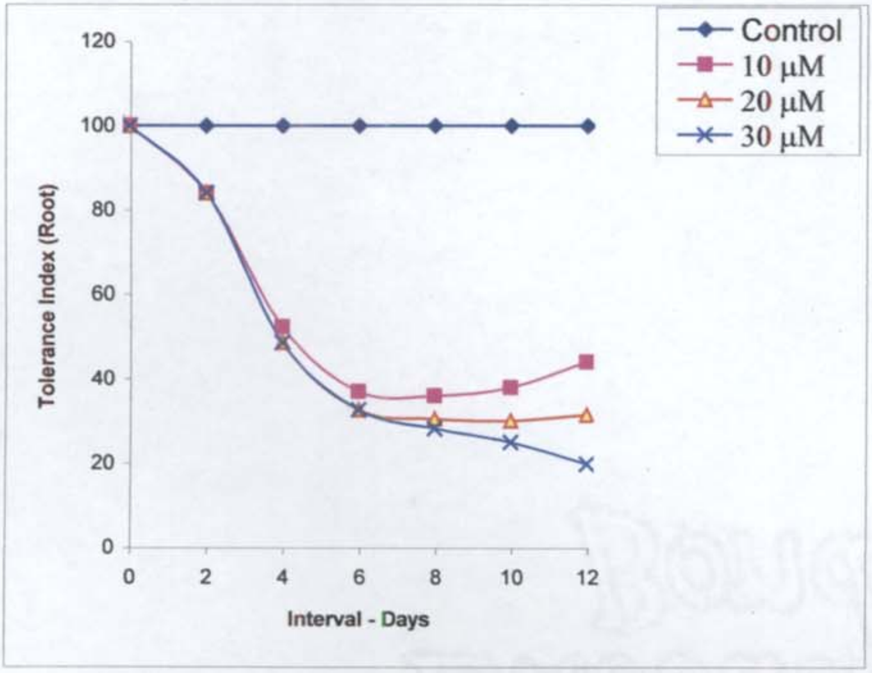
1. 4. Tolerance index percentage based on root length:

Plants treated with $CdCl_2$ showed sharp decline of tolerance index assessed based on root growth (Table 9, Fig. 12) up to 6 days of treatment in all concentrations. The tolerance index of 10 μM treatment was significantly increased during 10–12 days ($P<0.02$). But treatment with 20 μM $CdCl_2$ remained almost constant values with negligible increase and 30 μM treatment resulted in further decline of tolerance index.

Table: 9 Effect of Cadmium on root tolerance index percentage in *Bacopa monneiri* during growth.

Tissue	Treatment concentrations ($CdCl_2$)	Intervals - Days						
		0	2	4	6	8	10	12
Root	Control	100	100	100	100	100	100	100
	10 μM	100	84.04 ± 0.33	52.30 ± 0.24	36.98 ± 0.44	35.97 ± 0.31	37.97 ± 0.20	44.02 ± 0.04
	20 μM	100	84.04 ± 0.23	48.61 ± 0.23	32.64 ± 0.63	30.53 ± 0.22	30.06 ± 0.18	31.44 ± 0.09
	30 μM	100	84.04 ± 0.21	48.61 ± 0.22	32.64 ± 0.66	28.21 ± 0.21	25.00 ± 0.18	19.87 ± 0.03

Fig. 12 Effect of Cadmium on the root tolerance index percentage in *Bacopa monneiri* during growth.



1.5. Stomatal Index

Stomatal index value of *Bacopa monnieri* treated with 10 μM $CdCl_2$ showed only negligible increase in upper epidermis but significant increase in stomatal index value was shown by lower epidermis during 10–12 days of growth (Table 10). Treatment with 20 μM resulted in significant increase ($P<0.02$) of stomatal index value of upper epidermis compared to that of the lower epidermis after 8th day. Similarly during 8th, 10th and 12th days stomatal index value of both upper and lower epidermis of plants treated with 30 μM $CdCl_2$ exhibited significant increase compared to that of the other treatments and control (Table 10).

Table: 10 Effect of Cadmium on stomatal index percentage in *Bacopa monnieri* during growth.

Interval-Days	Control		Treatment concentrations ($CdCl_2$)					
	Upper epidermis	Lower epidermis	Upper epidermis			Lower epidermis		
	-	-	10 µM	20 µM	30 µM	10 µM	20 µM	30 µM
0	22.01 ± 1.46	20.00 ± 1.06	20.04 ± 0.16	21.79 ± 1.40	20.03 ± 1.00	24.03 ± 1.78	24.12 ± 2.01	27.20 ± 1.00
2	22.04 ± 0.94	20.98 ± 1.04	21.01 ± 0.91	20.98 ± 1.43	22.89 ± 1.91	21.14 ± 1.03	26.11 ± 2.00	26.20 ± 1.02
4	22.12 ± 0.85	21.68 ± 0.98	21.90 ± 0.81	20.48 ± 1.38	21.68 ± 1.90	20.01 ± 1.00	25.21 ± 1.81	27.23 ± 1.02
6	22.96 ± 0.16	22.73 ± 0.16	23.40 ± 0.23	20.21 ± 1.79	25.00 ± 1.91	22.00 ± 1.04	25.22 ± 1.70	26.19 ± 1.30
8	22.81 ± 0.81	20.65 ± 1.12	23.61 ± 0.16	27.92 ± 1.84	31.96 ± 1.92	24.00 ± 1.3	26.23 ± 1.82	28.12 ± 1.31
10	22.61 ± 1.00	21.42 ± 1.00	22.02 ± 0.18	32.82 ± 1.78	33.58 ± 1.89	23.03 ± 1.00	28.20 ± 1.81	38.09 ± 1.20
12	23.48 ± 1.10	21.95 ± 0.96	24.96 ± 0.31	38.06 ± 1.96	39.75 ± 1.94	28.05 ± 0.99	30.19 ± 1.90	37.19 ± 1.19

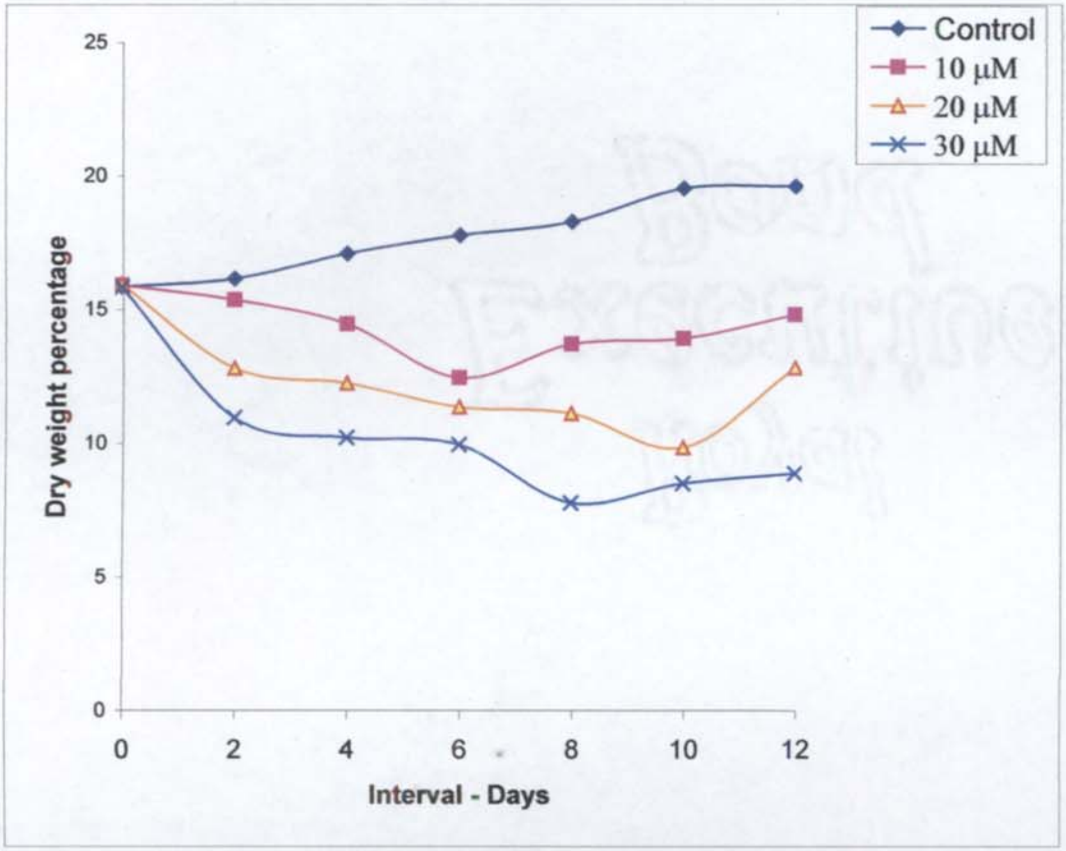
1.6. Dry weight percentage distribution

Dry matter content of *Bacopa monnieri* propagules treated with 10 µM $CdCl_2$ was significantly reduced compared to the control on 4th day and during further growth, dry weight percentage was gradually reduced (Table 11, Fig. 13). In propagules treated with 20 µM concentration also dry weight percentage was reduced significantly in comparison with the control and within the treatment gradual reduction was occurred during growth. Treatment of 30 µM $CdCl_2$ resulted in continuous decrease of dry matter throughout the experimental period and the dry weight values of all intervals were significantly reduced compared to other treatments and their respective controls.

Table: 11 Effect of Cadmium on dry weight percentage distribution in *Bacopa monnieri* during growth.

Interval Days	Control	Treatment concentrations ($CdCl_2$)		
		10 µM	20 µM	30 µM
0	15.85 ± 0.42	15.90 ± 0.68	15.93 ± 0.49	15.82 ± 0.59
2	16.15 ± 0.39	15.36 ± 0.58	12.80 ± 0.62	10.96 ± 0.59
4	17.09 ± 0.51	14.45 ± 0.47	12.24± 0.63	10.21 ± 0.64
6	17.77 ± 0.34	12.44± 0.48	11.35 ± 0.67	9.95 ± 0.63
8	18.27 ± 0.24	13.70± 0.51	11.11 ± 0.66	7.76 ± 0.64
10	19.53 ± 0.18	13.90 ± 0.55	11.83 ± 0.54	8.49 ± 0.54
12	19.61 ± 0.36	14.80 ± 0.54	12.81 ± 0.66	8.86 ± 0.64

Fig. 13 Effect of Cadmium on dry weight percentage distribution in *Bacopa monnieri* during growth.



2. ANATOMICAL/HISTOCHEMICAL STUDIES

2.1. Root

Microtome sections roots treated with 10, 20 and 30 μM concentrations of $CdCl_2$, stained with safranin showed thick and distorted piliferous layer in which dark deposits are seen. Stained masses are also seen in the xylem vessels of roots. Longitudinal sections of roots showed translocation of Cadmium as elongated patches/columns through the undifferentiated vascular tissues (Fig. 14a, A-D2).

Fig.14a

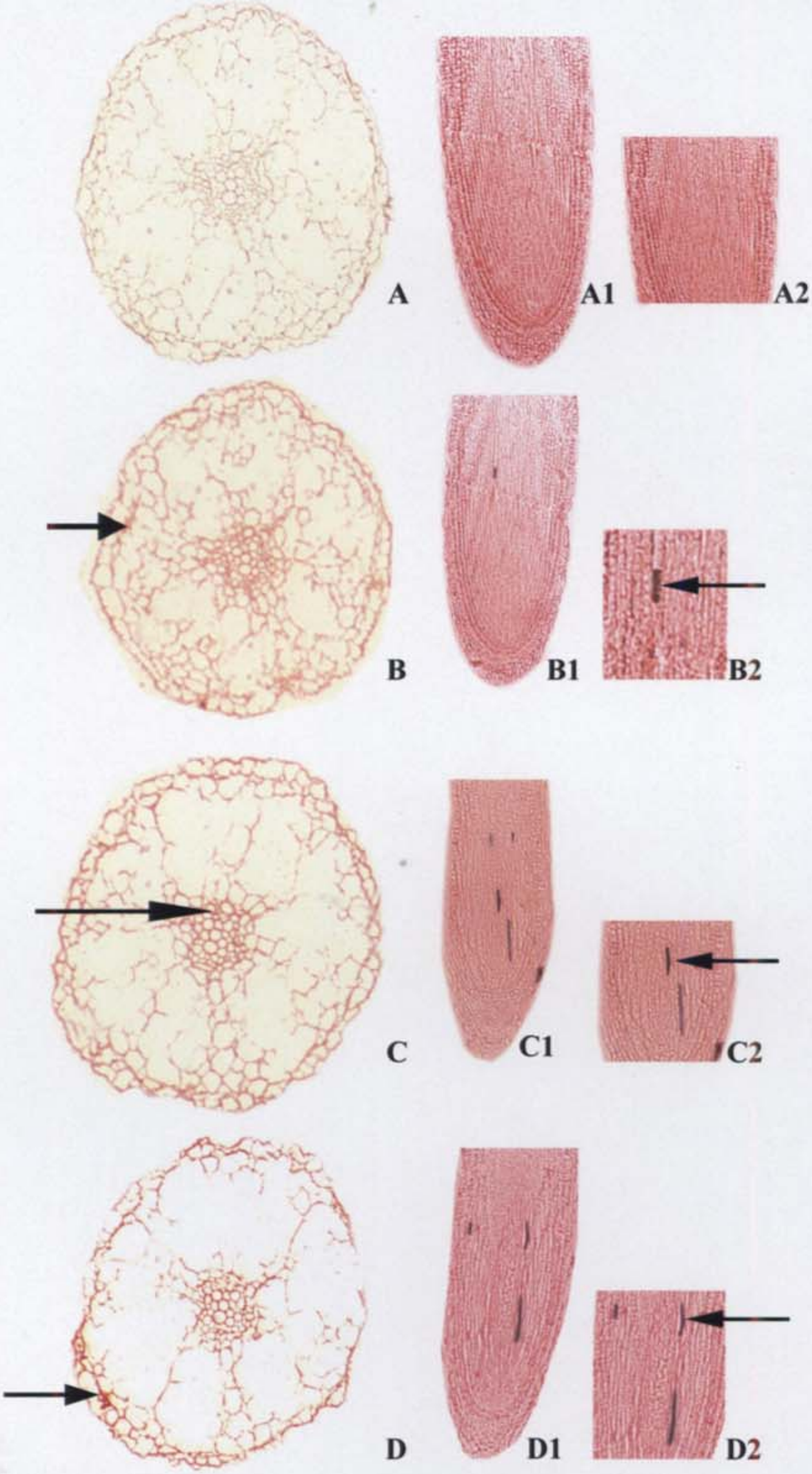


Fig. 14a- *Bacopa monnieri* treated with $CdCl_2$
A-D2 (Root)

A - Control (C.S)
A1- Control (L.S)
A2- Enlarged

B - 10 μM (C.S)
B1- 10 μM (L.S)
B2- Enlarged

C - 20 μM (C.S)
C1- 20 μM (L.S)
C2- Enlarged

D - 30 μM C.S
D1- 30 μM L.S
D2- Enlarged

Arrow denotes thick distorted cell walls/ Cd deposits

Fig. 14b

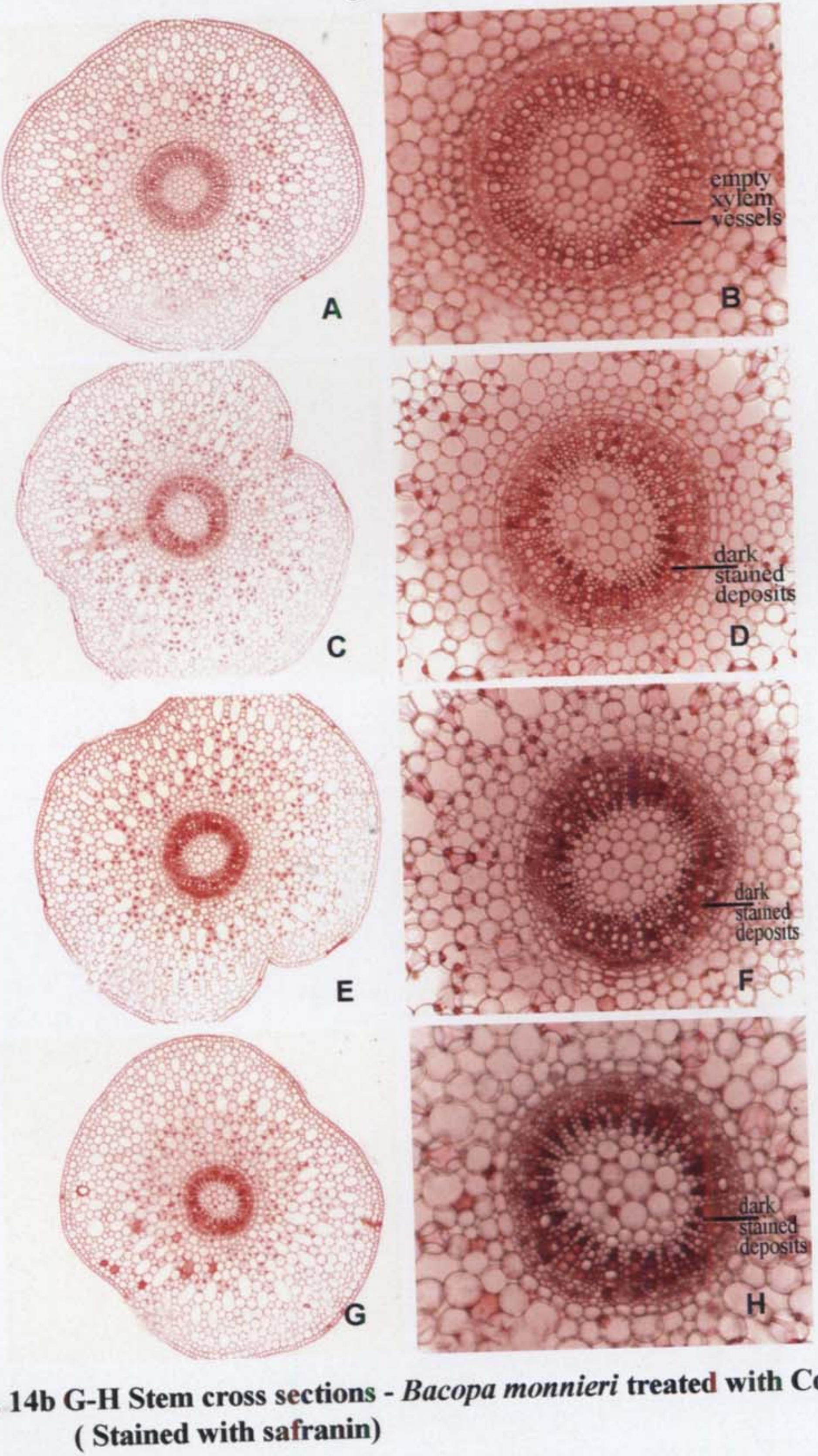


Fig. 14b G-H Stem cross sections - *Bacopa monnieri* treated with $CdCl_2$ (Stained with safranin)

A - Control - ground plan(Hoagland solution)
B - Enlarged stele
C -10 μM
D- Enlarged stele
E - 20 μM
F - Enlarged stele
G -30 μM
H - Enlarged stele

Fig. 14c

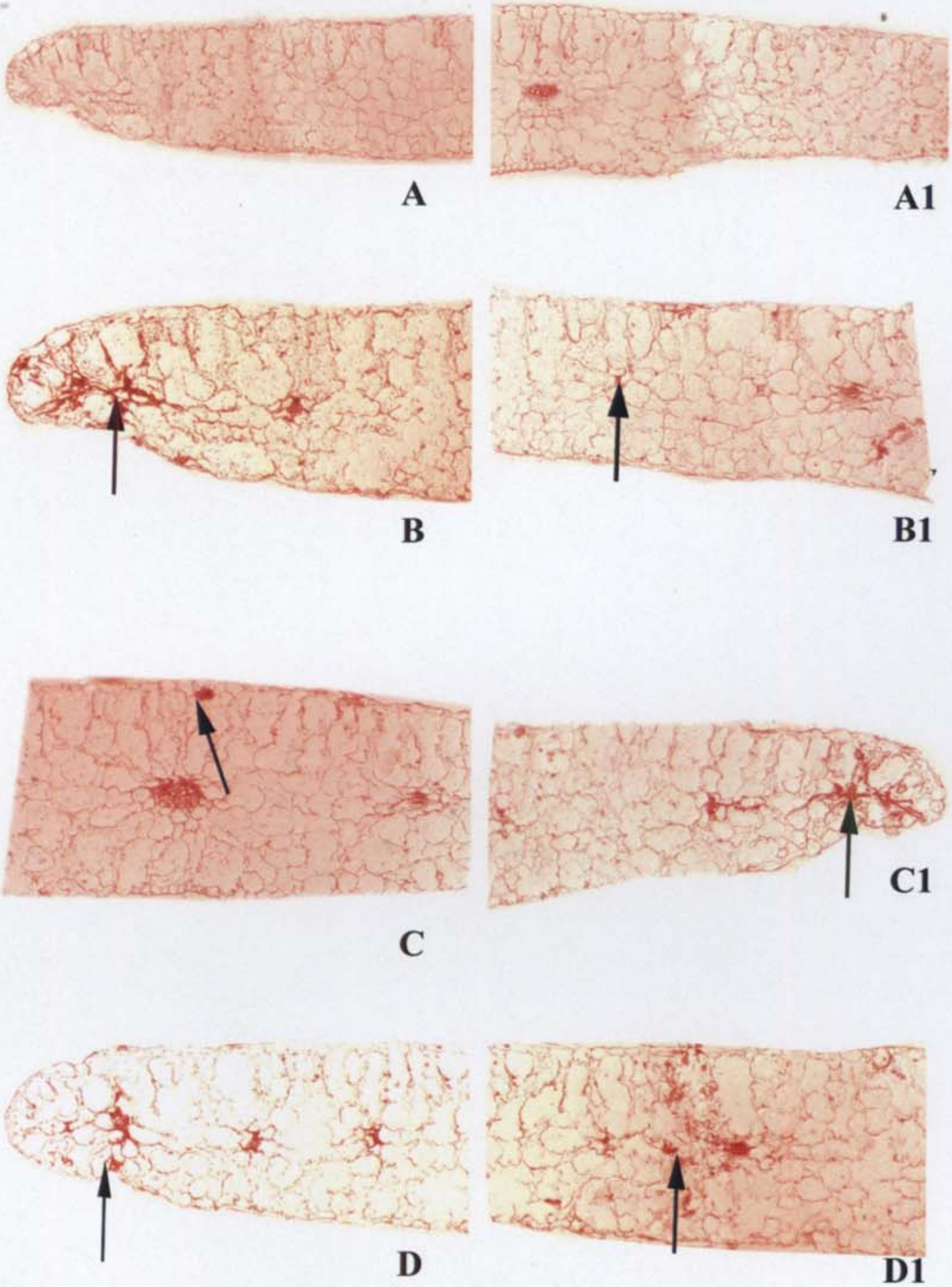


Fig. 14c *Bacopa monnieri* treated with $CdCl_2$
A-D1 (Leaf C.S)

A&A1- Control B&B1- 10 µM C&C1- 20 µM D &D1- 30 µM

Arrow denotes stained deposits of Cd

2.2. Stem

Anatomical details of stem tissue treated with $CdCl_2$ were similar to that of $HgCl_2$ treated plants. The free-hand sections of stem of propagules treated with $CdCl_2$ in 10 µM concentration exhibited the occurrence of safranin stained masses in the xylem vessels especially in the protoxylem (Fig. 14b, C&D). There occurred more stained masses filling whole protoxylem vessels and most of the metaxylem vessels in stem treated with 20 µM $CdCl_2$ (Fig. 14b, E&F). Similarly stem tissues of propagules treated with 30 µM $CdCl_2$ also showed the presence of stained masses in almost all (except 3-4 xylem vessels) protoxylem and metaxylem vessels (Fig. 14b, G&H). Localization of stained masses were absent in the pith, endodermis and cortex in all treatments. Orange brown coloured deposits in xylem vessels were also observed in the cross sections of stem of *B. monnieri* plants treated with $CdCl_2$ and stained with dithizone (Fig. 6c, K&L).

2. 3. Leaf

The microtome sections of leaves showed some stained patches near the leaf margins and surrounding the vascular bundles in the treatments of 10, 20 and 30 µM concentrations of $CdCl_2$, when stained with safranin which were absent in control leaf (Fig. 14c, A-D1).

3. PHYSIOLOGICAL AND BIOCHEMICAL STUDIES

3.1. Total protein

Protein content of propagules treated with 10, 20 and 30 µM $CdCl_2$ was decreased significantly compared to the control and all treatments contained more or less the same amount of protein (Table 12, Fig. 15) on 4th day. On 6th day, sharp decline of protein content was observed in 10 μM treatment whereas 20 μM treatment showed only slight reduction ($P<0.02$) and in 30 μM concentration, the protein content remained unchanged. Protein contents of 10 and 20 μM treatments were continuously and significantly increasing from 8th day to 12th day. Protein content of propagules of 30 μM treatment remained unchanged from 4th to 6th day and slight increase during 8th, 10th and 12th days was occurred.

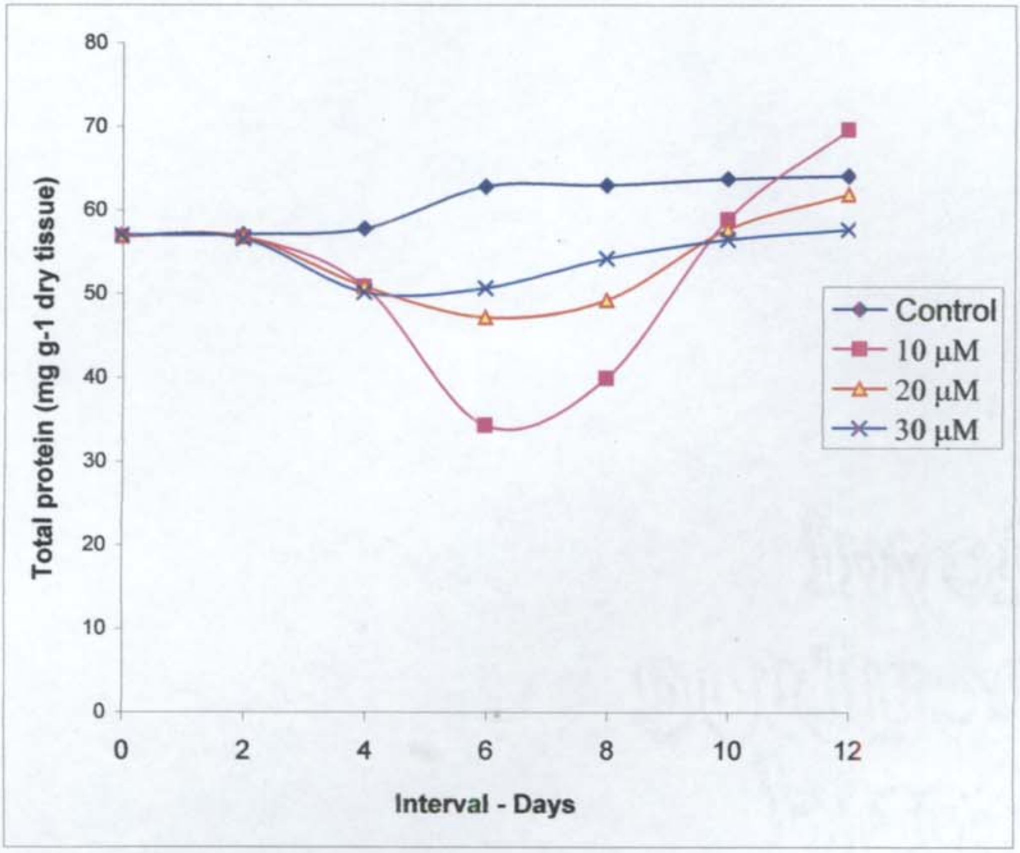
Table: 12 Effect of Cadmium on total protein content in *Bacopa monnieri* during growth.

(mg g^{-1} dry tissue)

Treatment concentrations ($CdCl_2$)	**Interval- Days**						
	0	**2**	**4**	**6**	**8**	**10**	**12**
Control	56.98 ± 0.49	57.08 ± 0.39	57.79 ± 0.61	62.79 ± 0.34	62.96 ± 0.24	63.74 ± 0.18	64.08 ± 0.36
10 μM	56.81 ± 0.36	56.73 ± 0.58	50.84 ± 0.63	34.26 ± 0.48	39.86 ± 0.51	58.87 ± 0.55	69.64 ± 0.54
20 μM	56.95 ± 0.54	56.77 ± 0.62	50.86 ± 0.64	47.14 ± 0.67	49.18 ± 0.66	57.66 ± 0.54	61.89 ± 0.66
30 μM	56.99 ± 0.51	56.54 ± 0.59	50.18 ± 0.66	50.66 ± 0.63	54.17 ± 0.64	56.44 ± 0.54	57.68 ± 0.64

Fig. 15 Effect of Cadmium on total protein content in *Bacopa monnieri* during growth.

(mg g^{-1} dry tissue)



3. 2. Protein profile (PAGE)

Electrophoretic separation patterns of protein profile showed more number of bands in 20 and 30 µM treatments of $CdCl_2$ resulting in elaborated number of bands of molecular weight between 47.2 and 79.8 kDa in comparison with the protein profile of control (Fig. 8). On 4th day, number of protein bands in the samples treated with both 20 and 30 µM $CdCl_2$ were more clear and distinct compared to the earlier stage. Additional thin bands were appeared below molecular weight of 47.2 kDa in these treatments. No significant variation was observed in the protein profile of plants treated with 20, 30 μM $CdCl_2$ on 6th and 10th days. In 8th day samples more distinct bands were shown as comparable to 4th day samples. But 12th day samples showed the absence of some distinct bands of molecular weight between 50.4 and 79.8 kDa. But additional thin bands were appeared towards lower region having molecular weight between 21 and 47.2 kDa.

3. 3. Chlorophylls

Leaves of *Bacopa monnieri* propagule treated with 10 μM of $CdCl_2$ showed a highly significant increase of Chl-a, b, total chlorophyll and a/b ratio after two days of treatment compared to the control (Table 13, Fig. 16). Up to 6th day the same trend with negligible increase of all the chlorophyll contents was observed. Chl-b content was significantly ($P<0.01$) increased on 8th day resulting in a slight increase in total chlorophyll but a significant reduction in a/b ratio ($P<0.02$). On 10th day, both Chl-a and b were increased with more enhancements in Chl-a maintaining the a/b ratio similar to the previous sample and almost the same trend was followed on 12th day with an increase in Chl-a compared to that of 10th day sample ($P<0.02$).

Bacopa monnieri propagules treated with 20 μM $CdCl_2$ resulted in significant increase of Chl-a, b, total chlorophyll and a/b values similar to that of 10 μM treatment on 2nd day followed by a significant reduction in a/b ratio due to the increase of Chl-b on 4th day of sampling (Table 13, Fig. 16). Sixth day samples showed further increase ($P<0.05$) in Chl-b resulting in a lowering of a/b value compared to the previous sample. After 6th day in all intervals both Chl-a and b contents were increased gradually maintaining a proportional a/b ratio. Since the increase of Chl-a was more in all samples ($P<0.02$, $P<0.05$, $P<0.02$ respectively) for 8th, 10th and 12th day samples, total chlorophyll also showed gradual significant ($P<0.01$) increase after 8th day throughout the experimental period.

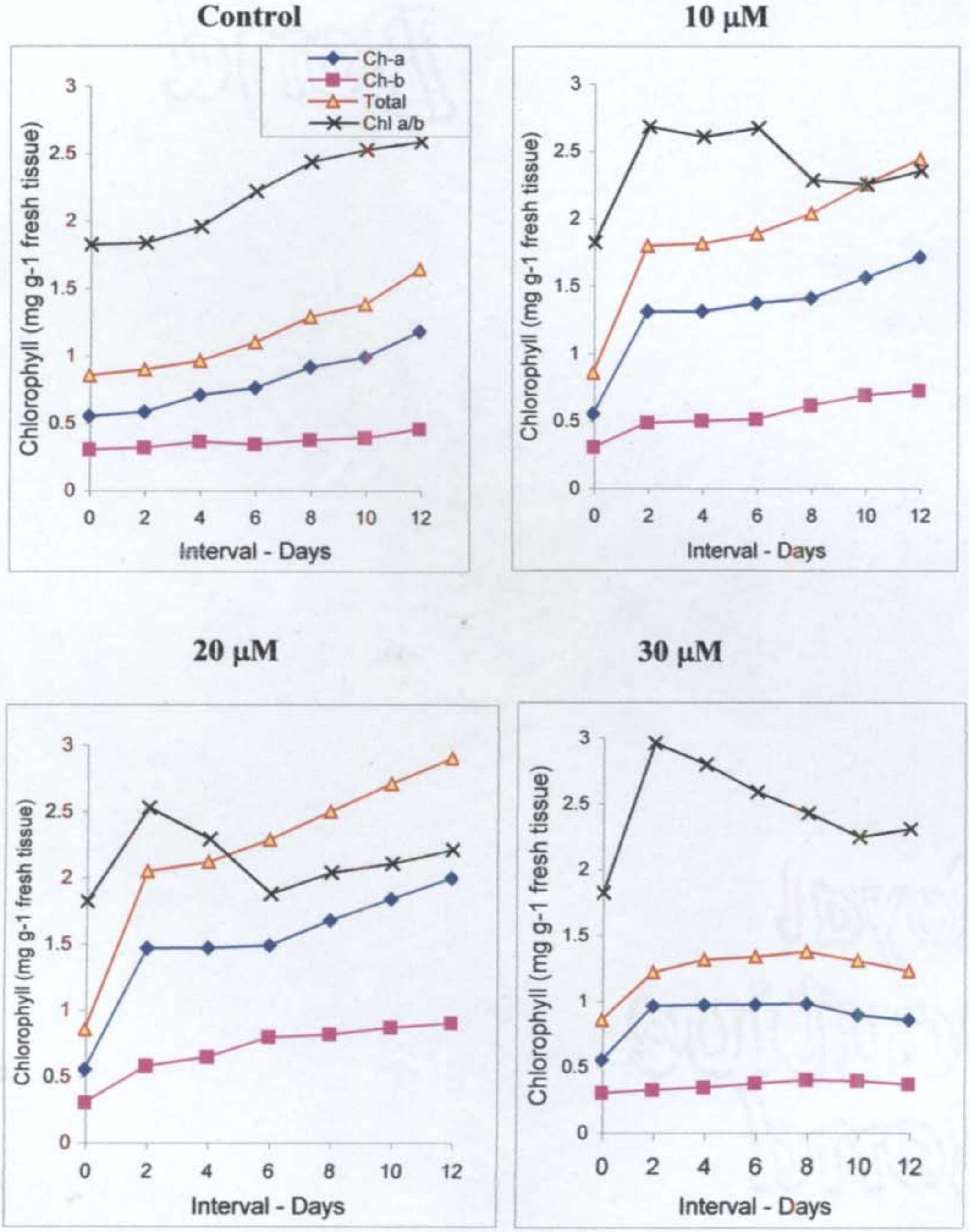
In *Bacopa monnieri* propagules treated with 30 μM $CdCl_2$, Chl-a and total chlorophyll contents were significantly increased on 2nd day of treatment. Only negligible increase in Chl-b was occurred resulting in a hike in the a/b ratio compared to control. Almost the same trend was followed up to 10th day with slight increase in Chl-b resulting in marginal increase of a/b ratio. Afterwards, both Chl-a, and b showed slight reduction resulting in a negligible fluctuation in chl-a/b ratio on 10th and 12th day samples (Table 13, Fig. 16).

Table: 13 Effect of Cadmium on chlorophyll content in *Bacopa monnieri* during growth.
(mg g^{-1} fresh tissue)

Treatment concentrations ($CdCl_2$)	Chlorophylls	Interval - Days						
		0	2	4	6	8	10	12
Control	Chl-a	0.55±0.01	0.58±0.01	0.71±0.01	0.76±0.02	0.91±0.03	0.98±0.04	1.18±0.04
	Chl-b	0.30±0.01	0.31±0.01	0.36±0.01	0.34±0.01	0.37±0.01	0.39±0.01	0.45±0.01
	Total	0.85±0.02	0.90±0.02	0.96±0.03	1.10±0.04	1.29±0.05	1.38±0.05	1.64±0.06
	Chl a/b	1.82±0.03	1.84±0.04	1.96±0.06	2.22±0.07	2.44±0.08	2.53±0.1	2.59±0.1
10 µM	Chl-a	0.55±0.02	1.31±0.03	1.31±0.03	1.37±0.03	1.41±0.04	1.56±0.04	1.71±0.05
	Chl-b	0.30±0.01	0.48±0.01	0.50±0.01	0.51±0.01	0.61±0.01	0.69±0.01	0.72±0.01
	Total	0.85±0.02	1.79±0.02	1.81±0.03	1.88±0.04	2.03±0.04	2.25±0.05	2.44±0.05
	Ch-a/b	1.82±0.03	2.68±0.04	2.60±0.04	2.67±0.04	2.28±0.03	2.25±0.04	2.35±0.04
20 µM	Ch-a	0.55±0.01	1.47±0.01	1.47±0.02	1.49±0.02	1.68±0.02	1.84±0.03	1.99±0.04
	Ch-b	0.30±0.01	0.58±0.01	0.64±0.01	0.79±0.02	0.82±0.02	0.87±0.02	0.90±0.02
	Total	0.85±0.02	2.05±0.02	2.12±0.02	2.28±0.03	2.50±0.03	2.71±0.04	2.90±0.05
	Ch-a/b	1.82±0.03	2.53±0.03	2.29±0.04	1.88±0.04	2.04±0.04	2.11±0.05	2.21±0.05
30 µM	Ch-a	0.55±0.02	0.96±0.02	0.97±0.02	0.97±0.02	0.98±0.02	0.89±0.03	0.86±0.04
	Ch-b	0.30±0.01	0.32±0.01	0.34±0.01	0.37±0.01	0.40±0.01	0.39±0.01	0.37±0.01
	Total	0.85±0.02	1.22±0.02	1.31±0.02	1.34±0.03	1.38±0.03	1.31±0.03	1.23±0.04
	Ch-a/b	1.82±0.03	2.96±0.04	2.80±0.04	2.59±0.04	2.43±0.04	2.25±0.04	2.31±0.05

Fig. 16 Effect of Cadmium on chlorophyll content in *Bacopa monnieri* during growth.

(mg g^{-1} fresh tissue)



4. BIOACCUMULATION STUDIES

4.1. Bioaccumulation of Cadmium (during 12 days treatments)

Accumulation of cadmium also was maximum in root tissue and order of accumulation was R>S>L (Table 14 a Fig. 17). Quantities of Cd accumulated in all tissues were increased proportional to the increase in concentration and progressed during growth period. Cadmium content accumulated in the root was double to the amount of stem and leaf tissues showed very low amount Cd content. Between the two concentrations of $CdCl_2$, 30 µM showed only marginal increase compared to 20 µM concentration (Table 14a, Fig. 17).

Cadmium showed very high accumulation in roots but the residual amount was considerably high showing gradual reduction as growth progressed. The quantity of Cd present in the residual medium was not much reduced during growth and hence the loss was not much elaborated (Table 14 b). When a comparison is made between Hg and Cd, absorption of Hg was more and hence lesser amounts were retained in residual medium but loss was significantly high. The Cd accumulation was significantly increasing but considerable amount was retained in the medium resulting in a reduced rate of loss during growth up to 12 days.

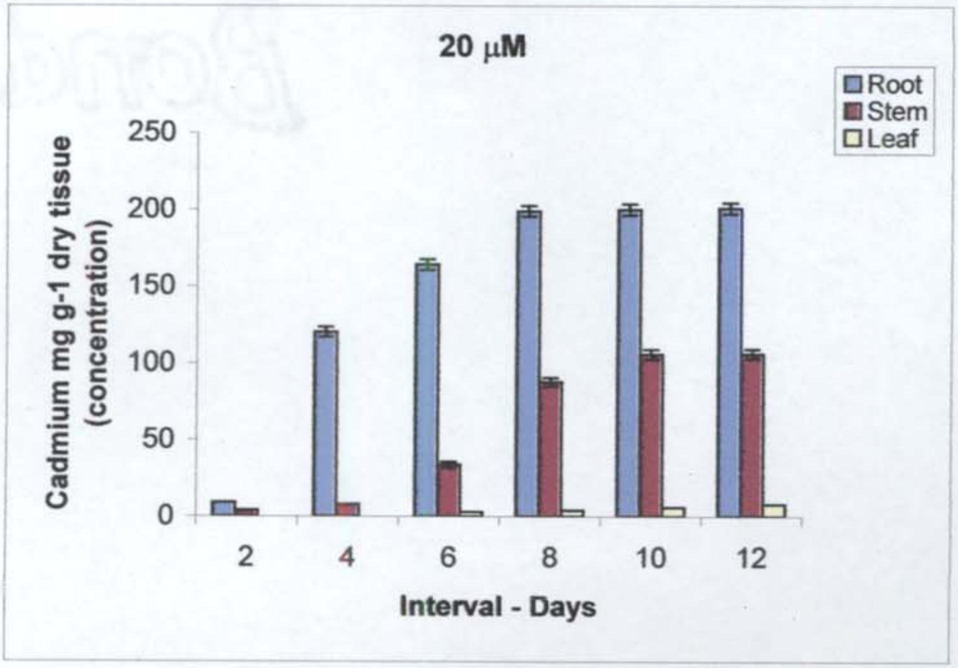
Table: 14a Accumulation of Cadmium contents in different parts of *Bacopa monnieri* treated with $CdCl_2$ during growth. mg g^{-1} dry tissue (concentration)

Concentrations		Plant parts	Interval-Days					
			2	4	6	8	10	12
$CdCl_2$	20 µM	Root	9.1± 0.03	120.1± 3.2	164.1± 3.5	199.0± 3.5	200.1± 3.6	200.9± 3.6
		Stem	4.0± 0.02	8.0± 0.03	34.0± 1.8	87.9± 2.5	105.8± 3.0	106.1± 3.0
		Leaf	NDR	2.0± 0.01	3.0± 0.01	4.0± 0.02	6.0± 0.02	8.0± 0.03
	30 µM	Root	12.7± 0.05	131.0± 3.2	230.0± 4.1	242.0± 4.2	238.0± 4.2	256.0± 4.4
		Stem	8.6± 0.04	8.0± 0.04	54.0± 2.5	100.0± 3.0	135.0± 3.3	187.0± 3.6
		Leaf	4.0± 0.01	6.0± 0.02	10.0± 0.04	13.0± 0.05	13.0± 0.05	15.0± 0.06

NDR- Non Detectable Range

Fig. 17 Accumulation of Cadmium contents in different parts of *Bacopa monnieri* treated with $CdCl_2$ during growth.

mg g^{-1} dry tissue (concentration)



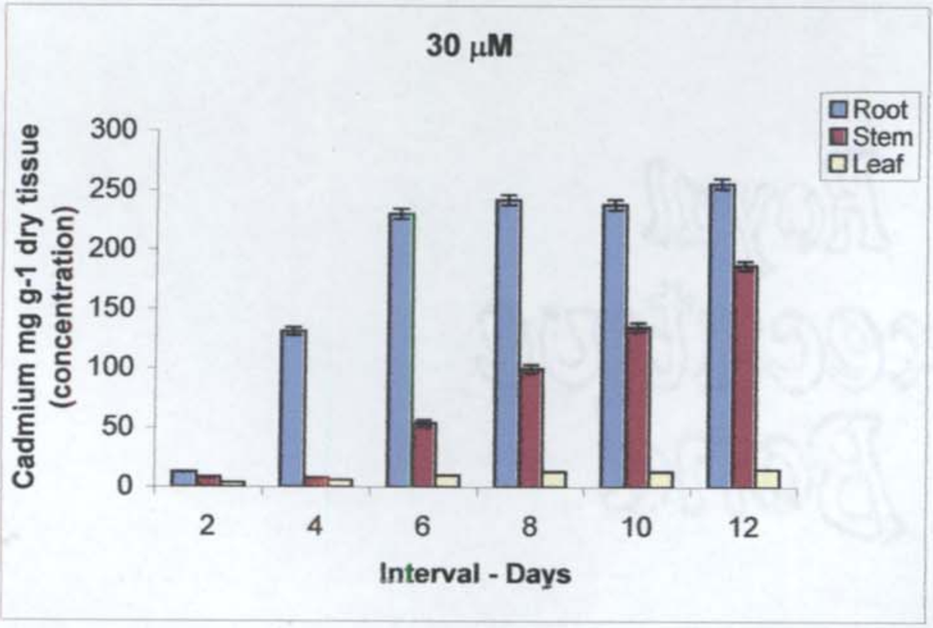


Table: 14b Percentage distribution of Cadmium in *Bacopa monnieri* in relation to the availability and loss during growth.
μg/ whole plants (content)

Treatment	Quantity given		Interval-Days					
			2	4	6	8	10	12
$CdCl_2$	**20 μM (448μg Cd)**	A	13 (2.9)	127 (28.3)	174 (38.8)	290 (64.7)	309 (68.9)	310 (69.1)
		R	415 (92.6)	310 (69.1)	201 (44.8)	109 (24.3)	69 (15.4)	60 (13.2)
		L	20 (4.4)	11 (2.4)	73 (16.2)	49 (10.9)	70 (15.6)	78 (17.4)
	30 μM (672μg Cd)	A	25 (3.7)	145 (21.5)	274 (40.7)	355 (52.8)	396 (58.9)	458 (68.1)
		R	606 (90.1)	457 (68.0)	328 (48.8)	234 (34.8)	168 (25.0)	94 (13.9)
		L	41 (6.1)	70 (10.4)	70 (10.4)	83 (12.3)	108 (16.0)	120 (17.8)

Values in parenthesis are percentage distributions

A - Total accumulation in plants (μg/whole tissue)

R - Residual content (μg) present in the medium during 12 days of growth

L - Quantity (μg) lost during 12 days growth (difference between accumulation + residue and total Cadmium content given

4. 2. Bioaccumulation of Cadmium (during 50 days treatment)

In 20 μM concentration of $CdCl_2$ treatment, bioaccumulation of Cd during 50 days growth, exhibited proportional increase to the concentration applied. But the percentage distribution values did not change significantly. Residual amount of Cd also was proportional to the concentration and percentage distribution did not show much variation. Loss of Cd exhibited slight increase during growth and percentage distribution remained unchanged (Table 14c).

Thirty μM concentration of $CdCl_2$, resulted in slight increase in the bioaccumulation pattern but distribution percentage did not vary. Residual amount of Cd also was proportional to the concentration and period of growth, but slight increase was observed in the percentage distribution. Loss of Cd showed only slight enhancement retaining the percentage distribution unchanged (Table 14c).

Table: 14c Bioaccumulation of Cadmium in *Bacopa monnieri* during repeated exposureof $CdCl_2$ up to 50 days

µg/whole plants (content)

			Interval-Days			
$CdCl_2$	20µM		20	30	40	50
		Quantity given				
			560 µg	672 µg	784 µg	896 µg
		A	364 (65.0)	404 (60.1)	432 (55.1)	508 (56.6)
		R	112 (20.0)	161 (23.9)	238 (30.3)	262 (29.2)
		L	84 (15.0)	107 (15.9)	114 (14.0)	126 (14.0)
	Quantity given					
	30µM		840 µg	1008 µg	1176 µg	1344 µg
		A	409 (48.6)	491 (48.7)	508 (50.3)	565 (49.9)
		R	275 (32.7)	353 (35.0)	480 (47.6)	574 (42.7)
		L	156 (18.5)	164 (16.2)	188 (18.6)	206 (15.3)

Values in parenthesis are percentage distributions

A - Total accumulation in plants (µg/whole tissue)

R - Residual content (µg) present in the medium, during 50 days of growth

L - Quantity (µg) lost during 50 days growth (difference between accumulation + residue and total Cadmium content given).

Table: 14d Relationship between BCF* and TF* in the bioaccumulation pattern of Hg and Cd in *Bacopa monnieri*

Treatments & concentrations			Interval -Days					
			2	4	6	8	10	12
$HgCl_2$	BCF	5 µM	0.19	0.70	1.07	1.46	1.95	3.30
		10 µM	0.10	0.20	0.21	0.27	0.35	0.61
	TF	5 µM	0.53	0.56	0.62	0.77	0.97	1.11
		10 µM	0.55	0.58	1.17	1.42	1.66	2.12
$CdCl_2$	BCF	20 µM	0.02	0.28	0.52	0.99	1.83	2.89
		30 µM	0.01	0.21	0.50	0.73	1.01	1.52
	TF	20 µM	0.44	0.08	0.22	0.45	0.55	0.57
		30 µM	1.00	0.10	0.27	0.46	0.62	0.78

*Ref**: Yoon *et al.*, (2006)
BCF - Bioconcentration factor (Accumulation ratio of growth medium to root)
TF - Translocation factor (Accumulation ratio of root to shoot)
Calculations based on the values of Tables 7a, b and 14a, b.

4.3. Effect of pH on the accumulation of Mercury and Cadmium in *Bacopa monnieri*

Effect of acidic (5.5) and alkaline (7.5) pHs showed very remarkable differences on metal accumulation (Table 15). At acidic pH, mercury translocation was more compared to control and two fold increases compared to the alkaline medium. Least amount of mercury was accumulated in plants grown in alkaline medium. In the case of Cd, the propagules grown in acidic medium accumulated more than 8 times in comparison with control whereas at alkaline medium, only very low quantity was accumulated (less than 1%). Combined treatment of $HgCl_2$ and $CdCl_2$ showed very low amount of Hg accumulation whereas only slight decrease of Cd was occurred compared to their individual treatment. Similarly, in alkaline and acidic medium, Hg accumulation showed negligible differences whereas Cd accumulation in the acid medium remained unchanged, but in alkaline medium resulted in exorbitant reduction of Cd accumulation (Table 15). When mercury treatment was given in Hoagland nutrient medium (pH 6.0) mercury accumulation was similar to that of the acidic pH whereas Cd content was significantly less than (only 27%) that of the acidic pH (Table 15).

Table: 15 Effect of pH on heavy metal uptake in *Bacopa monnieri* during 4 days of growth.

(µg g^{-1} dry tissue)

Treatment concentrations (10 µM)	Control (Distilled water) pH 6.8		Acidic medium pH 5.5		Basic medium pH 7.5		Hoagland solution pH 6.2	
$HgCl_2$ (400 µg Hg)	Hg	54.00± 0.03	Hg	68.88± 0.04	Hg	36.20± 0.02	Hg	66.30± 0.04
$CdCl_2$ (224 µg Cd)	Cd	35.46± 0.02	Cd	266.80± 0.07	Cd	2.39± 0.01	Cd	72.30± 0.04
$HgCl_2 + CdCl_2$ (400 µg Hg +224 µg Cd)	Hg	0.60± 0.01	Hg	0.47± 0.01	Hg	0.77± 0.01	Hg	0.60± 0.01
	Cd	23.98± 0.02	Cd	28.80± 0.02	Cd	1.02± 0.01	Cd	1.90± 0.01

5. SENSITIVITY OF *BACOPA MONNIERI* TOWARDS HEAVY METALS

5. 1. Natural habitat

Bioaccumulation of different heavy metals and their quantitative detection by using Atomic Absorption Spectrophotometer (AAS) in *Bacopa monnieri* plants, which were collected from different places in North Kerala, showed almost all samples with Al, As, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb and Zn (Table 16). *Bacopa monnieri* plants collected from Uppala (Kasaragod) which is notorious for indiscriminate use of endosulfan pesticides in cashew plantations contained maximum quantities of all the elements except Zn. Plants collected from Calicut city (sewage water) and Kanjikode (Industrial area) showed the occurrence of all elements more or less uniformly except Cd, Mn and Ni which were less in sewage water but more Cr, Hg and Zn were detected in Calicut city sewage water than Kanjikode industrial area. Plants collected from Mannuthy (Trissur) contained comparatively very low amount of some of the above mentioned elements while As, Cd, Hg and Ni were below the detection range (Table 16).

Table: 16 Bioaccumulations of various heavy metals in *Bacopa monnieri* collected from different places of North Kerala.

(mg g^{-1} dry tissue)

Samples	**Heavy metals detected**											
	Al	**As**	**Cd**	**Cr**	**Cu**	**Fe**	**Hg**	**Mn**	**Ni**	**Pb**	**Zn**	**Total**
Kanjikode (Palakkad)	0.201	0.002	0.398	0.440	0.188	0.808	0.021	0.224	0.100	1.211	0.212	3.805
City (Calicut)	0.280	0.002	0.100	0.644	0.100	0.818	0.049	NDR	0.010	1.234	0.436	3.673
Uppala (Kasaragod)	0.7006	0.1003	0.4248	0.4111	1.3487	2.0717	0.3149	0.2331	0.1004	1.8381	0.1482	7.6919
Mannuthy (Thrissur)	0.217	NDR	NDR	0.214	0.090	0.200	NDR	0.099	NDR	0.221	0.100	1.141

NDR- Non Detectable Range

5.2. Water samples of different locality of Calicut University Campus and nearby areas of Malappuram District

Quantitative detection of various heavy metals using Atomic Absorption spectrophotometer (AAS) revealed that, the tap water (Calicut University Water Supply) contained Al, As, Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn (Table 17 A). Lead and Fe occurred in higher quantities and As and Ni contents were very low while Hg was absent. Well water collected from a well in the campus showed the presence of all the elements mentioned above except Ni that was present in tap water, while Hg was present. In comparison with tap water, well water sample contained very low quantity of As, Cd and Fe. Bore well water contained very high quantities of all the elements in general, Cr, Cu, Fe and Pb in particular in comparison with well water or tap water. All elements mentioned above except Ni were present in sewage water collected from Calicut city, where as Cd and Hg contents were remarkably higher in this water sample. Effluents of Calicut University Water Treatment Plant showed the presence of all the elements, comparatively in moderate amounts. Water samples collected from a black stone quarry also contained more or less same elements in similar concentration compared to tap water. Water samples collected from two regions of Chaliyar River showed the occurrence of same elements but quantitatively significant differences were observed. Chaliyar river water (near Feroke area) was contaminated with industrial effluents and exorbitant amount of Al, Cd, Cr, Hg, Mn, Ni, Pb and Zn were present compared to all other water samples whereas the water samples collected from the same river (at Arekode region) showed the presence of all elements in very low quantities except Cu. Comparatively enhanced quantities of Cd, Cu, Hg, Mn, were present in water collected from paddy fields near to Calicut University Campus.

Marine water collected from Parappanangadi nearest coast of Calicut University contained all elements in which Cd, Cr, Fe, Hg, Mn and Pb contents were the most abundant quantities compared to all other water samples (Table 17 A).

Bacopa monnieri plants (rooted propagules) grown for 4 days in all the water samples as described earlier showed accumulations of safranin stained deposits in all the xylem vessels on free hand sections of stem (Fig. 18a, b) and AAS study reveals the presence of elements such as Al, As, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb and Zn and the quantitative distribution varied between water samples (Table 17A). When comparison is made between concentration of each metals present in water samples (mg l^{-1}) and that accumulated in *Bacopa monnieri* plants (mg g^{-1} tissue dry weight), the translocation of each element showed more or less uniform pattern *i,e.,* accumulation was proportional to metals available in the water samples (Tables 17B). Metals accumulated were calculated as content (quantity of each metal accumulated per total tissue grown in a known quantity of water containing known quantity of heavy metals and the ratio is represented as percentage (Table 17B). So it is observed that accumulation pattern of each metal also varied significantly. For example aluminium (Al) content of all water samples showed about 35-50% accumulation (Table 17 B) despite significant variations in the quantities present in water samples. But arsenic (As) did not show such uniform pattern of accumulation in *Bacopa* plant tissue. About 50% accumulation was shown by Cd whereas Cr accumulation pattern was not much uniform. Accumulation of Cu showed very high rate in almost all samples except water samples collected from paddy field. Mercury also showed variation in the rate of accumulation. Manganese, Ni, Pb and Zn did not show much variation, all were contained40-50% (Table 17B).

Table: 17A Distributions of different heavy metals in various local water

(mg l^{-1})

Water samples	**Heavy metals detected**										
	Al	**As**	**Cd**	**Cr**	**Cu**	**Fe**	**Hg**	**Mn**	**Ni**	**Pb**	**Zn**
Double Distilled Water (Control)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tap water	3.003	0.011	0.121	0.423	0.232	6.188	0.00	0.123	0.019	8.311	3.338
Well water	3.009	0.007	0.001	1.702	0.299	0.808	0.198	0.816	0.00	7.697	2.003
Bore well water	8.010	0.012	0.101	2.313	0.823	18.188	0.00	0.418	0.423	18.168	3.889
Rain water	1.018	0.007	0.098	1.811	0.111	6.444	0.104	0.00	0.00	8.887	0.00
Calicut city sewage water	7.321	0.087	1.009	1.972	0.301	7.298	2.102	1.737	0.00	8.297	2.998
Calicut University effluent water of Water Treatment Plant	6.136	0.081	0.201	0.810	0.418	9.342	0.020	0.313	0.181	4.101	5.050
Black stone quarry water	6.998	0.073	0.100	1.678	0.196	5.703	0.231	0.982	0.00	7.737	2.189
Chaliyar river water(Aecode area)	1.890	0.004	0.091	1.001	1.810	3.423	0.017	0.712	0.013	2.331	6.243
Chaliyar river water (Feroke area)	16.648	0.432	0.032	7.116	0.152	2.056	1.516	2.748	3.030	28.564	16.012
Paddy field water	4.120	0.008	1.018	1.001	3.434	7.469	3.243	3.243	0.096	14.326	4.001
Marine water	0.532	0.536	4.004	8.032	0.804	27.52	3.944	3.944	2.061	40.44	12.032

Table: 17B Bioaccumulations of various heavy metals in *Bacopa monnieri* cultivated in different local water ($mg\ g^{-1}$ dry tissue)

Water Samples	Heavy metals detected										
	Al	**As**	**Cd**	**Cr**	**Cu**	**Fe**	**Hg**	**Mn**	**Ni**	**Pb**	**Zn**
Double distilled water (Control)	0.00 (-)	0.00 (-)	0.00 (-)	0.00 (-)	0.00 (-)	0.00 (-)	0.00 (-)	0.00 (-)	0.00 (-)	0.00 (-)	0.00 (-)
Tap water	0.428 (35.6)	0.002 (45.4)	0.024 (49.5)	0.084 (49.6)	0.084 (90.5)	1.218 (49.2)	NDR (-)	0.060 (81.3)	0.030 (52.6)	1.618 (48.6)	0.648 (48.5)
Well water	0.428 (35.5)	NDR (-)	NDR (-)	0.25 (36.7)	0.25 (41.8)	0.104 (32.1)	0.032 (40.4)	0.162 (49.6)	NDR (-)	1.498 (48.6)	0.248 (30.9)
Bore well water	1.478 (46.1)	0.002 (41.6)	0.006 (14.8)	0.262 (28.3)	0.262 (79.5)	2.026 (27.8)	NDR (-)	0.082 (49.0)	0.044 (26.0)	3.034 (41.7)	0.640 (41.1)
Rain water	0.200 (49.1)	NDR (-)	0.004 (10.2)	0.142 (19.6)	0.142 (49.5)	0.992 (38.4)	0.016 (38.4)	NDR (-)	NDR (-)	1.444 (40.6)	NDR (-)
Calicut city sewage water	1.23 (42.0)	0.016 (45.9)	0.196 (48.5)	0.19 (24.0)	0.19 (91.3)	0.978 (33.5)	0.212 (25.2)	0.218 (31.3)	NDR (-)	1.48 (44.5)	0.58 (48.3)
Effluent of Water Treatment Plant of Calicut University	1.05 (42.7)	0.016 (49.3)	0.022 (27.3)	0.16 (49.3)	0.16 (95.6)	1.838 (49.1)	0.002 (25.0)	0.060 (47.9)	0.03 (41.4)	0.76 (46.3)	0.846 (41.8)
Black stone quarry water	1.2 (42.8)	0.014 (49.2)	0.020 (50.0)	0.164 (24.4)	0.164 (56.1)	0.71 (31.1)	0.016 (17.3)	0.192 (48.8)	NDR (-)	1.158 (37.4)	0.426 (48.6)
Chaliyar river water (Arecode area)	0.346 (45.7)	NDR (-)	0.016 (43.9)	1.98 (49.4)	1.98 (27.3)	0.642 (46.8)	0.002 (29.4)	0.14 (49.1)	0.002 (38.4)	0.448 (48.0)	1.228 (49.1)
Chaliyar river water (Feroke area	3.2 (48.0)	0.024 (13.8)	0.006 (46.8)	1.156 (40.6)	1.156 (92.1)	0.220 (26.7)	0.106 (17.4)	0.536 (48.7)	0.412 (33.9)	5.148 (45.0)	1.786 (27.8)
Paddy field water	0.802 (48.6)	NDR (-)	0.200 (49.1)	1.98 (49.4)	1.98 (14.4)	1.446 (48.4)	0.014 (1.0)	0.624 (48.1)	0.018 (46.8)	2.7 (47.1)	0.774 (48.3)
Marine water	0.104 (48.8)	0.006 (2.79)	0.616 (38.4)	0.860 (26.7)	0.860 (80.8)	5.41 (49.1)	0.032 (2.0)	0.724 (45.8)	0.402 (48.7)	5.668 (35.0)	1.988 (41.3)

Values in parenthesis are percentage distributions NDR-Non Detectable Range

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Fig. 18a

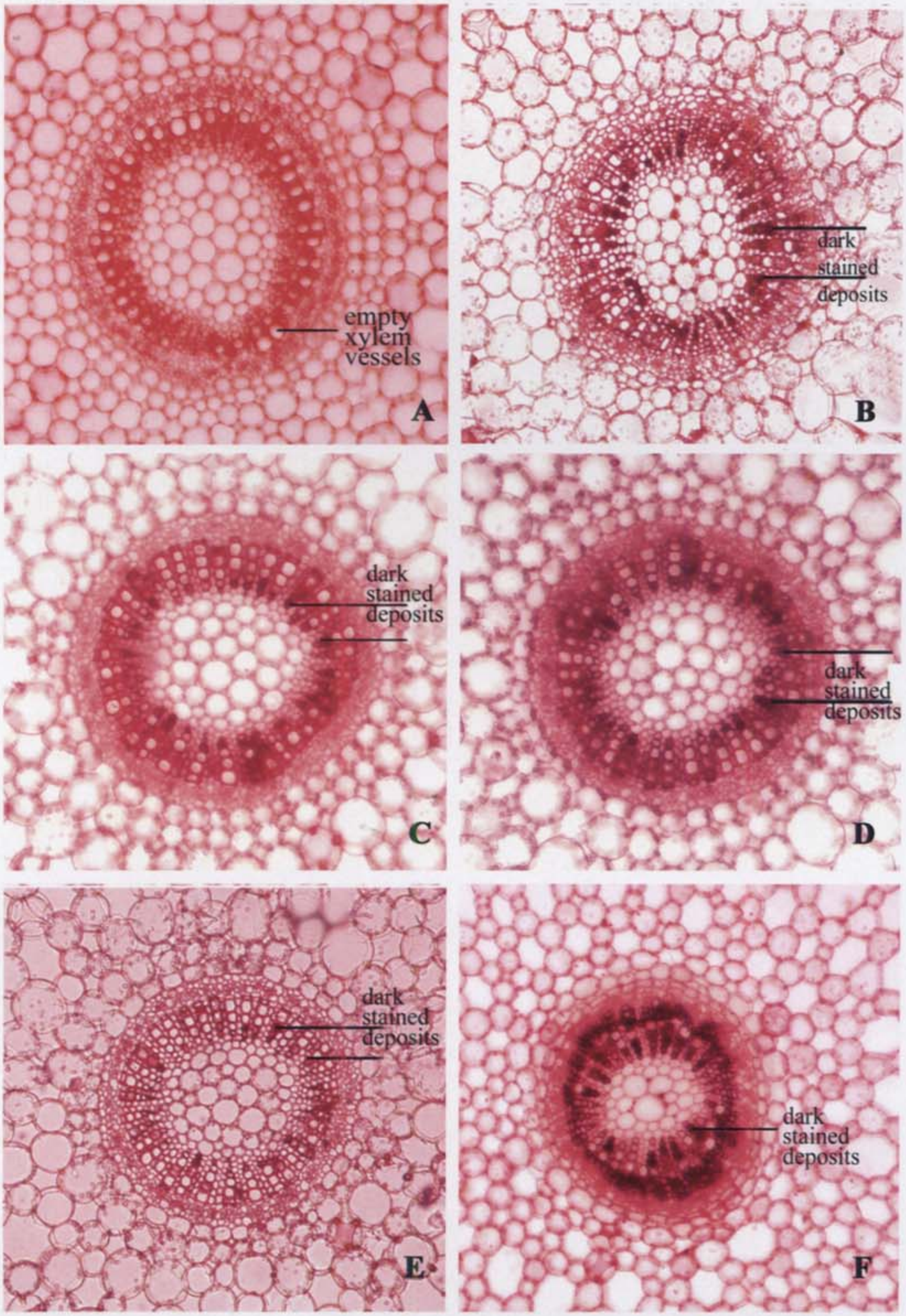

Fig. 18a Stem cross sections- *Bacopa monnieri* cultivated in various water

A- Distilled water	**D- Bore well water**
B- Tap water	**E- Rain water**
C- Well water	**F- Calicut city sewage water**

Fig. 18b

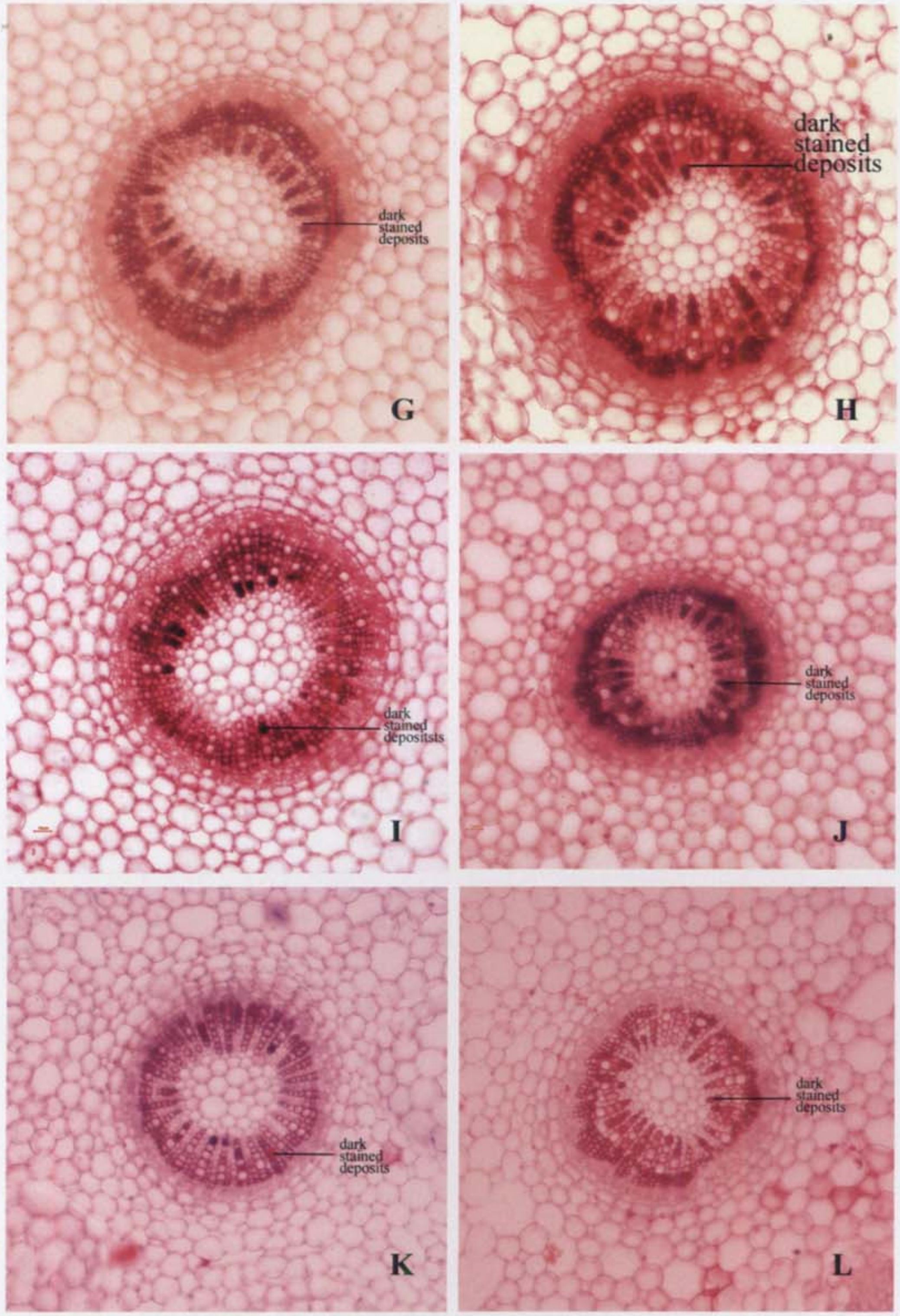

Fig. 18 b Stem cross sections- *Bacopa monnieri* cultivated in various water

G- Effluent water from Calicut University Water Treatment Plant
H- Black stone quarry water I- Chaliyar river water (Areecode area)
J- Chaliyar river water (Feroke area)
K- Paddy field water L- Marine water

5.3. Different commercially available mineral water samples

Several commercially available branded namely Aquafina, Aquaspring, King fisher, Omkar, Soorya, Surabhi and Lehar pepsi (Club soda) mineral water samples were detected using AAS analytical method and found that all contained Al, As, Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn in considerable quantities (Table 18A). Significant variations in the quantities of each metal were observed among different branded mineral water. Heavy metals such as As, Cd and Cr were present in all mineral water, tested. Kingfisher contained all elements except Hg and Ni. Mercury was present only in Omkar (Packaged drinking water), which contained maximum Cd, Cu, Fe, Ni and Pb. Zinc was comparatively low. Soorya and Surabhi also showed the presence of all the elements, except Hg in considerable quantities. All elements except Hg and Ni were present in Lehar Pepsi, only in very low quantity in comparison with other mineral water samples.

Bacopa monnieri plants cultivated in mineral water as mentioned earlier , showed the accumulation of all the elements (Table 18B, Fig. 19) were calculated as content (*i.e.*, total content of each heavy metal accumulated in a known weight of propagules cultivated in a known quantity of mineral water). The quantity of each metal entered the tissue varied depending upon their occurrence in the mineral water in which the plants were grown (Table 18 B).

Accumulation range of Al in all mineral water samples except Aquafina and Lahar Pepsi were 35–50% of the content present in the medium (Table 18 B). Arsenic, Cd, Cr, Fe and Mn accumulation was about 31–50% with some exceptions such as Cu and Fe accumulation was very feeble in the plants cultured in Aquaspring. Nickel, Pb and Zn accumulation showed wide fluctuation in accumulation rate among the mineral water samples. Plants cultured in Aquaspring showed 95% Pb accumulation. Similarly Zn accumulation was very low in the plants cultured in Aquafina.

Table: 18A Distributions of different heavy metals in various commercially available 'mineral water' ($mg\ l^{-1}$)

Samples (Trade Names) with B. No & Mfdt.	Heavy metals detected										
	Al	As	Cd	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Zn
Aquafina (9ND175/05-05) (Packaged drinking water)	1.00	0.012	0.759	4.410	2.041	10.285	0.00	1.370	0.494	6.990	1.003
Aquaspring (D27/09-03) (Packaged drinking water)	1.05	0.00	0.482	2.84	1.07	3.84	0.00	3.25	0.04	1.21	4.02
Kingfisher (299/08-03) (Packaged drinking water)	1.989	0.011	0.801	2.310	0.404	0.632	0.00	0.243	0.00	0.931	3.183
Omkar (184/01-05) (Packaged drinking water)	1.482	0.042	1.784	2.945	2.761	11.249	0.089	0.013	0.981	6.984	2.451
Soorya (232/05-06) (Packaged drinking water)	2.208	0.011	1.733	5.808	1.002	7.035	0.00	1.096	0.00	1.328	0.701
Surabhi (09/06-05) (Packaged drinking water)	4.881	0.011	1.308	4.915	0.603	3.407	0.00	0.700	0.00	0.00	4.180
Lehar Pepsi (NJ009/02-05) (Club Soda)	0.120	0.011	0.868	4.705	1.001	6.418	0.00	1.176	0.00	3.841	0.980

Table: 18B Bioaccumulation of various heavy metals in *Bacopa monnieri* cultivated in different commercially available 'mineral water'

(mg g^{-1} dry tissue)

Samples (Trade Names)	Heavy metals detected										
	Al	**As**	**Cd**	**Cr**	**Cu**	**Fe**	**Hg**	**Mn**	**Ni**	**Pb**	**Zn**
Aquafina (Packaged drinking water)	NDR (-)	0.002 (41.7)	0.128 (42.1)	0.81 (45.9)	0.35 (42.3)	2.054 (49.9)	NDR (-)	0.24 (43.7)	0.094 (47.5)	0.612 (21.8)	0.006 (1.49)
Aquaspring (Packaged drinking water)	0.162 (38.5)	NDR (-)	0.046 (23.8)	0.248 (21.8)	0.068 (15.8)	0.168 (10.9)	NDR (-)	0.482 (37.0)	0.002 (12.5)	0.462 (95.4)	0.206 (12.8)
Kingfisher (Packaged drinking water)	0.396 (49.8)	0.002 (45.4)	0.152 (47.4)	0.402 (43.5)	0.066 (40.8)	0.108 (42.7)	NDR (-)	0.042 (43.2)	NDR (-)	0.162 (43.5)	0.428 (33.6)
Omkar (Packaged drinking water)	0.248 (41.8)	0.008 (47.6)	0.356 (49.8)	0.588 (49.1)	0.426 (38.5)	1.81 (40.2)	0.016 (0.97)	0.002 (38.4)	0.196 (49.4)	1.198 (42.8)	0.500 (50.9)
Soorya (Packaged drinking water)	0.298 (33.7)	0.002 (45.4)	0.15 (21.6)	1.04 (44.7)	0.192 (47.9)	1.214 (43.1)	NDR (-)	0.136 (31.0)	NDR (-)	0.204 (38.4)	0.122 (43.5)
Surabhi (Packaged drinking water)	0.728 (37.2)	0.002 (45.4)	0.260 (49.6)	0.867 (44.5)	0.114 (45.2)	0.604 (44.3)	NDR (-)	0.138 (4.92)	NDR (-)	NDR (-)	0.632 (37.7)
Lehar Pepsi (Club Soda)	0.004 (8.3)	0.002 (45.4)	0.136 (39.1)	0.818 (43.4)	0.16 (4.0)	1.218 (47.4)	NDR (-)	0.234 (49.7)	NDR (-)	0.686 (44.6)	0.220 (56.1)

Values in parenthesis are percentage distributions   NDR-Non Detectable Range

Fig. 19

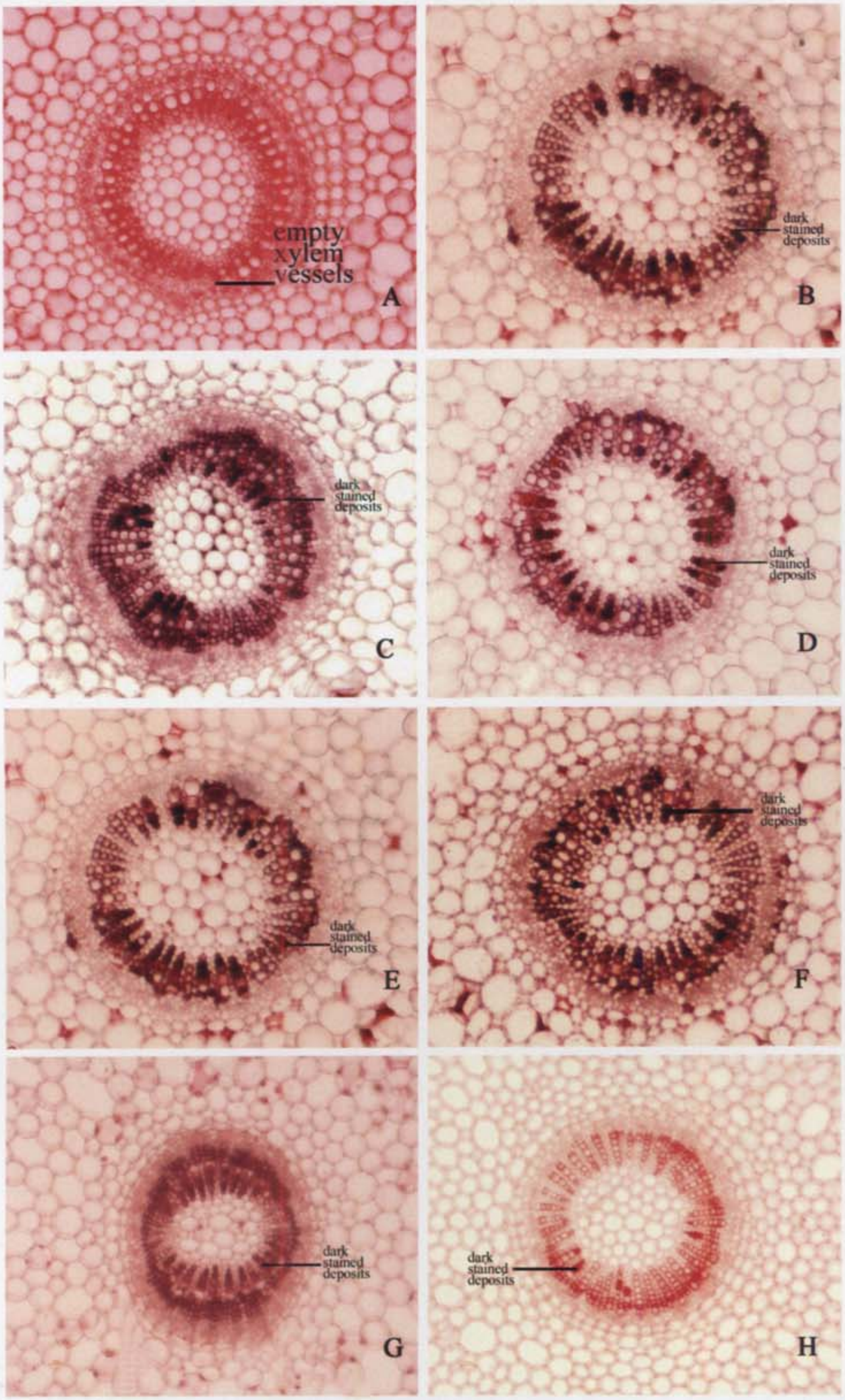

Fig. 19 Stem cross sections - *Bacopa monnieri*cultivated in various mineral water

A- Distilled water
B- Aquafina
C- Aquaspring
D- Kingfisher
E- Omkar
F- Soorya
G- Surabhi
H- Leharpepsi

5.4. Different commercially available 'soft drink' samples

Commercially available soft drinks such as Pepsi, CocaCola, 7-up, Mirinda, Fanta, Sprite, Thums up, Maaza, Limca and Slice showed the presence of Al, As, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb and Zn (Table 19 A) when subjected to AAS study. Aluminium content showed wide variation among different soft drinks. Maximum Al was present in Slice. Arsenic content was comparatively very low except CocaCola, which contained 5 mg l^{-1}. Cadmium was absent in Miranda, Fanta and Sprite, but very high in Pepsi and Slice. Very high quantity of Cr was presents in all soft drinks that are in the range of 20-25 mg l^{-1}. Pepsi, CocaCola, Sprite and Mirinda showed more amount of Cu than other soft drinks. Iron (Fe) content also showed slight variation in their occurrence. Pepsi, CocaCola, 7-up and Slice contained about 35 mg l^{-1}. Mercury was absent in Pepsi, CocaCola and Limca and maximum Hg was in Slice and 7-up. Distribution of Mn showed wide variation showing maximum that is 10 mg l^{-1} in 7- up, Sprite and Slice. Nickel was present only in Maaza. All soft drinks, except Fanta and Sprite, showed the presence of very high Pb content (50–85 mg l^{-1}). Maximum amount of Zn was present in Slice and Maaza and Fanta contained only very small quantity of Zn.

Bioaccumulations of the heavy metals in *Bacopa monnieri* cultivated in these soft drinks were more or less comparable to the accumulation of mineral water (Table 19B, Fig. 20a,b). Aluminium concentration was more in plants cultivated in Pepsi and Fanta (45–46%) and very low in plants of Maaza and sprite (Table 19 B). Plants cultivated in CocaCola showed more arsenic (As) than other soft drinks. Cadmium concentration was in the range of 31-49% except in Mirinda, Fanta and Sprite, where accumulation was not detected. More or less uniform accumulation (40-50%) of Cr was shown by plants cultivated in all the soft drinks.

Plants cultivated in different soft drinks showed significant variation in the accumulation of Cu. Maximum accumulation of Cu was in the plants grown in CocaCola, Pepsi and Thums up and Slice showed very feeble concentration of Cu. Iron accumulation was more or less uniform in the range of 41–58% except in Slice. Bioaccumulation of Hg in *Bacopa monnieri* plants showed 25-50% accumulation except in the plants grown in 7-Up, which contained 0.8 mg/l Hg, but accumulation was not in detectable range. Manganese content concentration was in the range of 32-50% except Pepsi and CocaCola showed 4% and 18% respectively. Eventhough Ni was present in Maaza (0.25 mgl^{-1}) this metal was not accumulated in *Bacopa monnieri* plants grown in all soft drinks, showed the presence of Pb and Zn in the range of 27-51% and 24-48% respectively.

Table: 19A Distributions of different heavy metals in various commercially available 'soft drinks' (mg l^{-1})

Samples (Trade Names) with B.No&Mfdt.		Heavy metals detected										
		Al	As	Cd	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Zn
Pepsi	(436/08-06)	0.94	0.05	4.45	23.99	3.83	35.49	NDR	5.10	NDR	66.80	10.94
Coca Cola	(142/07-06)	1.50	5.05	0.86	23.25	4.01	34.41	NDR	4.40	NDR	85.60	11.20
7-up	(06/06-06)	11.10	0.20	0.80	24.66	NDR	35.18	0.80	10.00	NDR	84.95	10.04
Mirinda	(418/05-05)	11.06	0.20	NDR	24.06	4.53	30.72	0.30	6.50	NDR	64.60	9.92
Fanta	(107/04-06)	7.51	0.06	NDR	23.75	0.87	28.13	0.21	7.01	NDR	0.26	2.40
Sprite	(192/06-05)	5.02	0.06	NDR	21.73	3.55	10.40	0.24	10.85	NDR	7.24	2.44
Thums-up	(140/07-05)	17.40	0.03	1.35	24.45	0.60	16.72	0.45	1.51	NDR	51.00	12.20
Maaza	(434/04-06)	5.00	0.30	1.06	20.61	0.20	25.06	0.455	4.50	0.25	48.09	15.56
Limca	(162/02-06)	11.65	0.01	0.61	20.50	2.02	30.07	NDR	0.81	NDR	61.71	4.74
Slice	(214/09-06)	19.32	0.400	5.06	25.61	2.65	35.70	0.91	10.05	NDR	66.20	19.32

NDR-Non Detectable Range

Table: 19 B Bioaccumulations of various heavy metals in *Bacopa monnieri* cultivated in different commercially available 'soft drinks'

(mg g^{-1} dry tissue)

Samples (Trade Names)	Heavy metals detected										
	Al	As	Cd	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Zn
Pepsi	0.034 (44.9)	0.002 (50.0)	0.156 (43.8)	0.808 (42.2)	0.14 (45.6)	1.186 (41.7)	NDR (-)	0.016 (3.9)	NDR (-)	2.096 (39.2)	0.420 (47.9)
CocaCola	0.028 (23.2)	0.01 (2.47)	0.022 (31.9)	0.500 (48.3)	0.156 (48.6)	1.186 (43.0)	NDR (-)	0.062 (17.6)	NDR (-)	2.056 (30.0)	0.246 (24.4)
7-up	0.244 (27.4)	0.008 (20.0)	0.03 (46.8)	0.922 (46.7)	NDR (-)	1.29 (45.1)	NDR (-)	0.382 (47.7)	NDR (-)	2.01 (29.5)	0.368 (45.8)
Mirinda	0.31 (35.0)	0.006 (36.5)	NDR (-)	0.938 (48.7)	0.106 (29.2)	1.032 (41.9)	0.006 (24.5)	0.242 (46.5)	NDR (-)	1.401 (27.1)	0.284 (35.7)
Fanta	0.282 (46.9)	0.002 (41.6)	NDR (-)	0.93 (48.9)	0.020 (28.7)	0.928 (41.2)	0.006 (35.7)	0.268 (47.7)	NDR (-)	0.008 (38.4)	0.076 (39.5)
Sprite	0.032 (7.9)	0.002 (38.4)	NDR (-)	0.872 (50.1)	0.03 (10.5)	0.358 (43.0)	0.008 (41.6)	0.282 (32.4)	NDR (-)	0.208 (35.9)	0.076 (38.9)
Thums-up	0.448 (32.1)	NDR (-)	0.036 (33.2)	0.808 (41.3)	0.022 (4.4)	0.664 (49.6)	0.018 (50.0)	0.046 (38.0)	NDR (-)	2.078 (50.8)	0.284 (29.0)
Maaza	0.03 (7.4)	0.002 (8.1)	0.028 (33.0)	0.802 (48.6)	0.004 (24.3)	0.846 (42.1)	0.016 (43.9)	0.178 (49.4)	NDR (-)	1.922 (49.9)	0.600 (48.1)
Limca	0.284 (30.4)	NDR (-)	0.022 (44.7)	0.810 (49.1)	0.068 (42.0)	1.002 (41.6)	NDR (-)	0.024 (36.8)	NDR (-)	2.228 (45.1)	0.086 (22.6)
Slice	0.488 (31.5)	0.004 (12.5)	0.200 (49.3)	0.826 (40.3)	0.002 (0.94)	0.264 (9.20)	0.032 (43.9)	0.402 (49.9)	NDR (-)	2.268 (42.8)	0.488 (31.5)

Values in parenthesis are percentage distributions NDR-Non Detectable Range

Fig. 20a

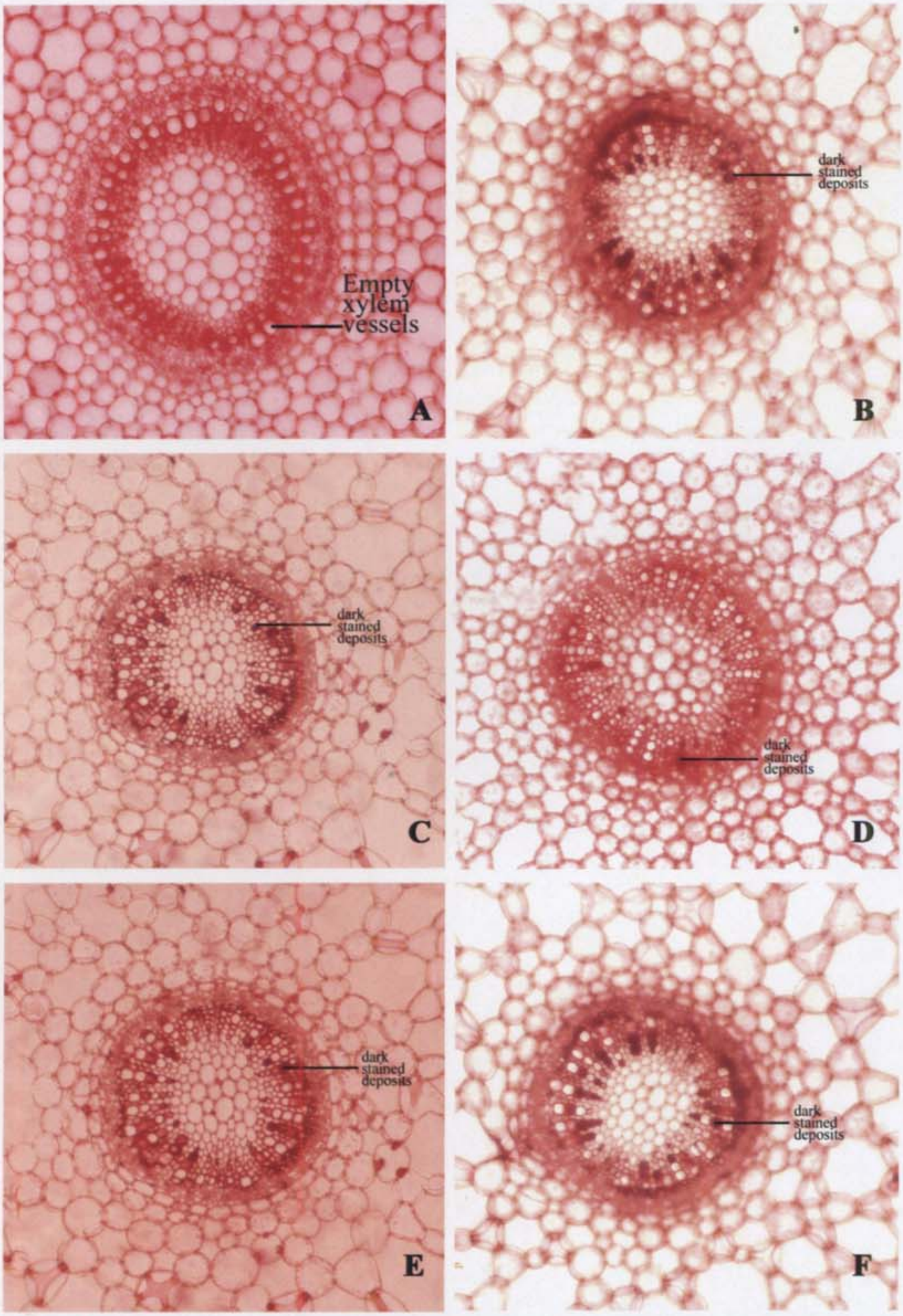

Fig. 20a Stem cross sections in *Bacopa monnieri* cultivated in various soft drink

A- Distilled water	D- 7 Up
B- Pepsi	E- Mirinda
C- CocaCola	F- Fanta

Fig. 20b

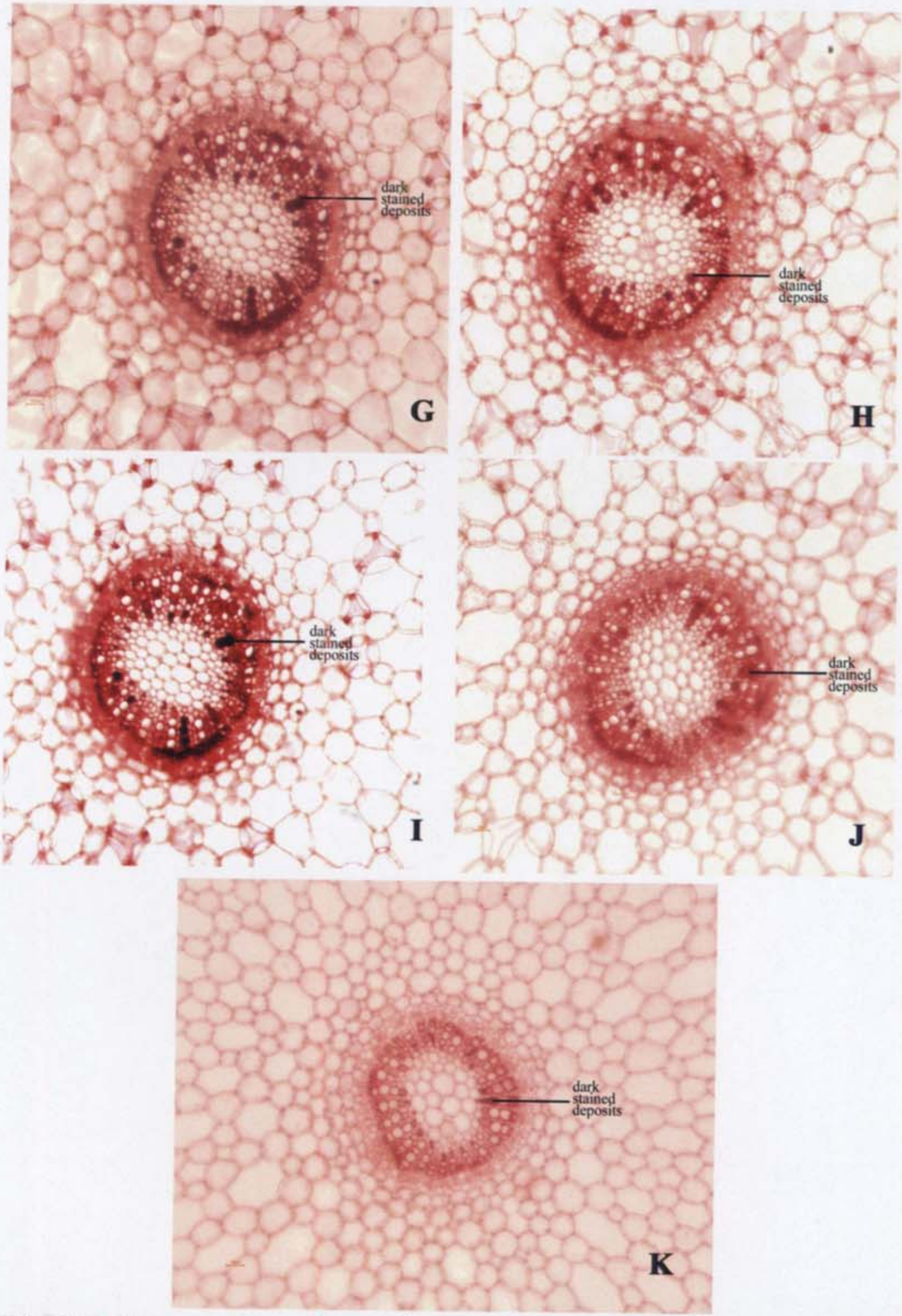

Fig. 20b Stem cross sections - *Bacopa monnieri* cultivated in various soft drinks

G- Sprite
H- Thums Up
I- Maaza
J- Limca
K- Slice

5.5. Different commercially available 'fruit juice' samples

Fruit juices namely Appy, Frooti, Rasna and Tang when used to AAS study, found that all of them contained considerable quantities of heavy metals such as Al, As, Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn (Table 20 A). Mercury was absent in all fruit juices. Aluminium contents of Rasna and Tang were very high while Appy and Frooti contained only very low amount. Arsenic was absent in Appy and Frooti where as negligible in Rasna and Tang. Frooti was devoid of Cd but Tang contained comparatively more Cd. Chromium content was maximum in Rasna and Tang while Frooti and Appy contained only negligible quantity. Similarly Cu also was more in Rasna and Tang, Fe was less in Frooti. Manganese, Pb and Zn content were maximum in Tang and least amount in Appy. Nickel was present in Tang and Appy, Frooti with neligible amount but was not detectable range in Rasna. Zinc was devoid in Frooti.

Bacopa monnieri plants cultivated in fruit juices showed the accumulation of all the heavy metals present in the fruit juice samples (Table 20B, Fig. 21). Aluminium accumulation was proportional to the content of all fruit juice brands. Eventhough As was present in trace quantities in Rasna and Tang, the accumulation was not observed in *Bacopa monnieri*. Accumulation of Cd was very high, up to 95 and 98% of the total available Cd was accumulated in the plant when grown in Rasna and Tang respectively (Table 20 B). Almost the same pattern of accumulation was noted in Cr and Cu. Tang showed maximum concentration of Mn. But in Rasna, Mn accumulation was very high. Frooti showed only 35 % accumulation. Eventhough Ni was present in Appy, Frooti and Tang, accumulation was observed only in plants cultivated in Tang (20%). Zinc content was 90% and 78% respectively in the plants grown in Rasna and Tang and the accumulated quantity was not proportional to the amount of the metals present in the juices. Bioaccumulation of Pb was 100% in plants grown in Rasna which contained lesser quantity of Pb compared to Tang. Lead content accumulated in the plants cultivated in Appy and Tang also were not proportional to their content occurred in their respective juice samples.

Table: 20A Distributions of different heavy metals in various commercially available 'Fruit juices'

(*mg l^{-1} , **mg g^{-1})

Samples (Trade Name) with B.No&Mf.dt.	Heavy metals detected										
	Al	As	Cd	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Zn
Appy* (Apple) (B/0706)	0.40	NDR	0.16	2.04	0.50	1.71	NDR	0.22	0.14	2.89	0.745
Frooti *(Mango) (10BC/05-06)	0.28	NDR	NDR	2.40	0.25	0.2	NDR	1.27	0.06	3.21	NDR
Rasna**(Orange) (112/01-06)	10.35	0.02	0.60	13.87	1.835	22.81	NDR	0.62	NDR	10.6	2.78
Tang** (Lemon) (--/04-06)	10.03	0.03	1.48	11.16	2.03	21.84	NDR	6.91	0.27	17.98	5.00

NDR-Non Detectable Range

Table: 20B Bioaccumulations of various heavy metals in *Bacopa monnieri* cultivated in different commercially available 'fruit juices'

(mg g^{-1} dry tissue)

Samples (Trade Name)	**Heavy metals detected**										
	Al	**As**	**Cd**	**Cr**	**Cu**	**Fe**	**Hg**	**Mn**	**Ni**	**Pb**	**Zn**
Appy (Apple)	0.014 (43.2)	NDR (-)	0.006 (46.8)	0.05 (30.6)	0.012 (29.7)	0.006 (4.30)	NDR (-)	0.004 (22.7)	NDR (-)	0.066 (28.4)	0.016 (26.8)
Frooti (Mango)	0.004 (17.5)	NDR (-)	NDR (-)	0.044 (26.8)	0.006 (30.0)	0.008 (50.0)	NDR (-)	0.036 (35.2)	NDR (-)	0.68 (26.4)	NDR (-)
Rasna (Orange)	0.642 (77.4)	NDR (-)	0.046 (95.0)	1.092 (98.4)	0.14 (95.3)	0.806 (98.9)	NDR (-)	0.048 (96.7)	NDR (-)	0.846 (99.7)	0.200 (89.7)
Tang (Lemon)	0.568 (70.7)	NDR (-)	0.116 (97.6)	0.836 (93.6)	0.154 (94.8)	1.625 (94.5)	NDR (-)	0.216 (39.0)	0.022 (20.0)	1.192 (82.8)	0.312 (77.9)

Values in parenthesis are percentage distributions NDR-Non Detectable Range

Fig.21

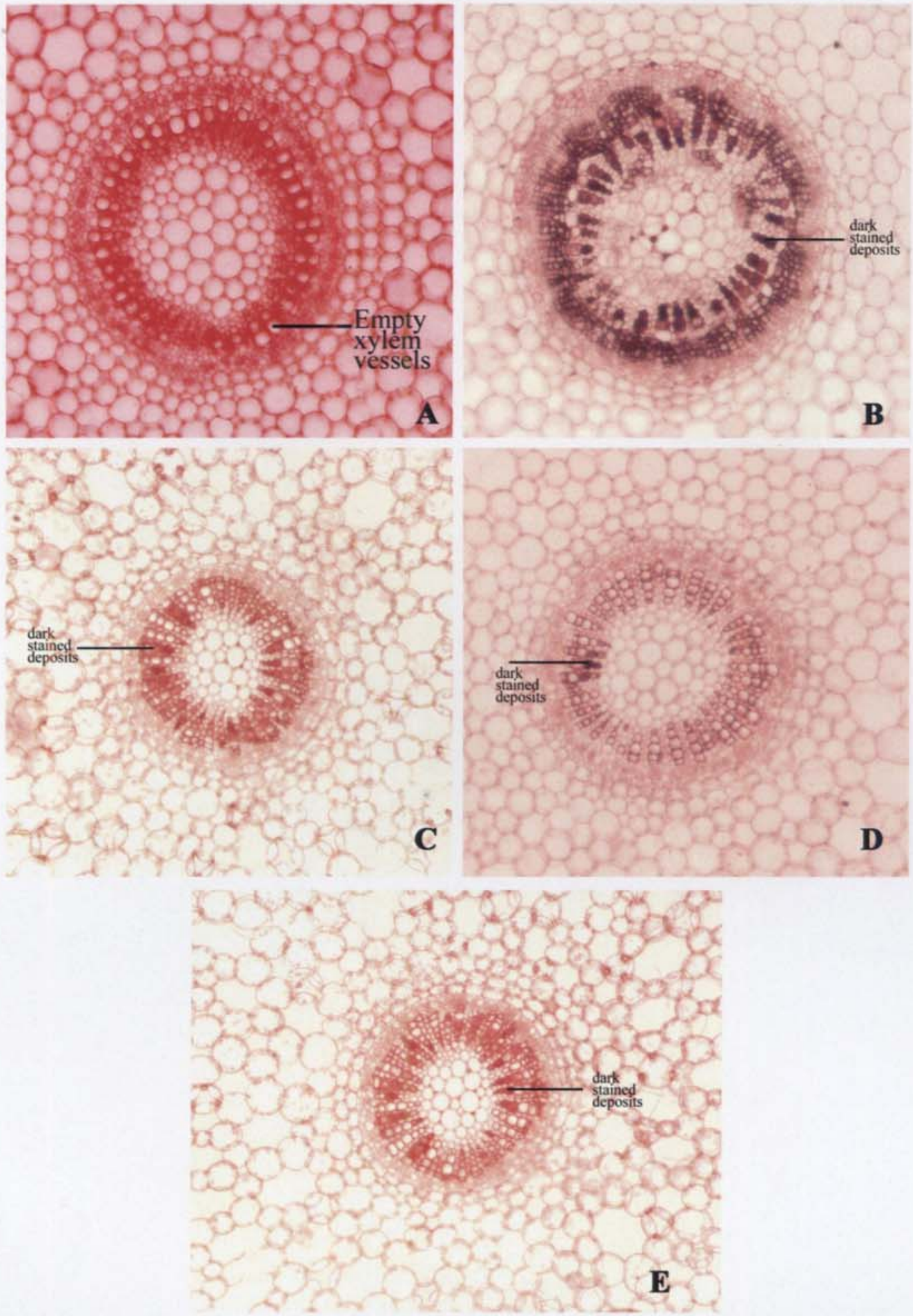

Fig.21 Stem cross sections- *Bacopa monnieri* cultivated in various fruit juices

A- Distilled water
B- Appy
C- Frooti
D- Rasna
E- Tang

5.6. Different commercially available 'Brahmi product' samples

Heavy metals such as Al, As, Cd, Cr, Cu, Fe, Hg, Mn, Pb and Zn were present in all products of Brahmi when quantitative detection of those metals were done by AAS (Table 21 A). Brahmi vita, Brahmi rich and Brahmi plus capsules are available in granules. Aluminium, Cd, Cr, Fe and Mn contents were more in Brahmi plus capsules compared to Brahmi vita and Brahmi rich, while Hg was very low in these products. Jyothish Brahmi and Brahmi syrup which are liquid products contained all heavy metals except As. Nickel was under non detectable range in Brahmi syrup. Aluminium, Cu, Fe and Mn were more in Brahmi syrup where as Cd, Cr, Ni, Pb and Zn were more in Jyothish Brahmi. Nickel was present only in Jyothish Brahmi.

Bioaccumulation of heavy metals in *Bacopa monnieri* when cultivated in the medium of Brahmi products showed accumulation of almost all metals present in the Brahmi products. Aluminium content was more in Brahmi syrup and As did not accumulate in plants considerably of any Brahmi products. Cadmium content was proportional to the content of the products (Table 21B, Fig. 22). Accumulation of Cr was very high (30-77%) in all products and maximum accumulation was in plants grown in Brahmi plus capsules. Copper content was comparatively not much but their accumulation range was above 29% except Brahmi plus capsules. In Brahmi plus capsule it was 9.7%. Accumulation of Fe was almost proportional to the content present in the medium. Accumulation of Fe was only 48% even when the Fe content of Brahmi syrup was 21mg l^{-1}. Eventhough Hg content was below to the detectable ranges in *Bacopa monnieri* grown in all Brahmi products, manganese content accumulated was not proportional to the quantity present in the product. Nickel was detected only in Jyothish Brahmi and accumulation was 46%. Iron accumulation was maximum in plants grown in Brahmi vita (61%), where as Jyothish Brahmi contained very high content of Zn (Table 21 B) but accumulation was very feeble (1%).

Table: 21A Distributions of different heavy metals in various commercially available 'Brahmi products'

(*mg g^{-1} ,mg l^{-1})**

Samples with B.No &Mf.dt.	Heavy metals detected										
	Al	As	Cd	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Zn
Brahmi Vita* (B68507 Dec-2005)	0.035	0.031	0.00	0.334	0.617	0.400	0.003	0.270	NDR	5.812	0.435
Brahmi Rich* (D7….05-2005)	0.045	0.035	0.00	0.193	0.165	0.640	0.004	0.396	NDR	4.332	0.894
Brahmi plus capsule* (041-No-326)	0.079	0.002	0.159	0.602	0.075	2.090	NDR	0.593	NDR	4.776	0.375
Jyothish Brahmi** (2/11-06)	0.46	NDR	2.96	7.06	1.72	6.115	0.06	6.09	4.55	17.155	11.6
Brahmi syrup** (012/10-05)	3.99	NDR	2.05	1.09	6.38	20.98	0.01	8.865	NDR	15.62	9.35

NDR-Non Detectable Range

Table: 21B Bioaccumulations of various heavy metals in *Bacopa monnieri* cultivated in different commercially available 'Brahmi products'

(mg g^{-1} dry tissue)

Samples (Trade Name)	Heavy metals detected										
	Al	**As**	**Cd**	**Cr**	**Cu**	**Fe**	**Hg**	**Mn**	**Ni**	**Pb**	**Zn**
Brahmi Vita	NDR (-)	0.008 (64.5)	NDR (-)	0.156 (44.1)	0.106 (42.9)	0.098 (61.2)	NDR (-)	0.032 (29.6)	NDR (-)	0.552 (23.7)	0.08 (45.9)
Brahmi Rich	0.002 (11.1)	NDR (-)	NDR (-)	0.036 (46.6)	0.026 (39.3)	0.074 (28.9)	NDR (-)	0.004 (2.5)	NDR (-)	0.328 (19.9)	0.200 (55.9)
Brahmi plus capsule	0.006 (18.9)	NDR (-)	0.014 (22.0)	0.186 (77.2)	0.03 (9.7)	0.200 (23.9)	NDR (-)	0.07 (29.5)	NDR (-)	0.208 (10.8)	0.094 (62.6)
Jyothish Brahmi	0.01 (27.1)	NDR (-)	0.108 (45.5)	0.172 (30.4)	0.04 (29.0)	0.182 (37.2)	NDR (-)	0.112 (22.9)	0.168 (46.1)	0.592 (43.1)	0.048 (1.00)
Brahmi syrup	0.162 (50.6)	NDR (-)	0.076 (46.2)	0.036 (41.2)	0.21 (41.1)	0.81 (48.20	NDR (-)	0.274 (38.6)	NDR (-)	0.636 (50.8)	0.294 (39.20

Values in parenthesis are percentage distributions NDR-Non Detectable Range

Fig. 22

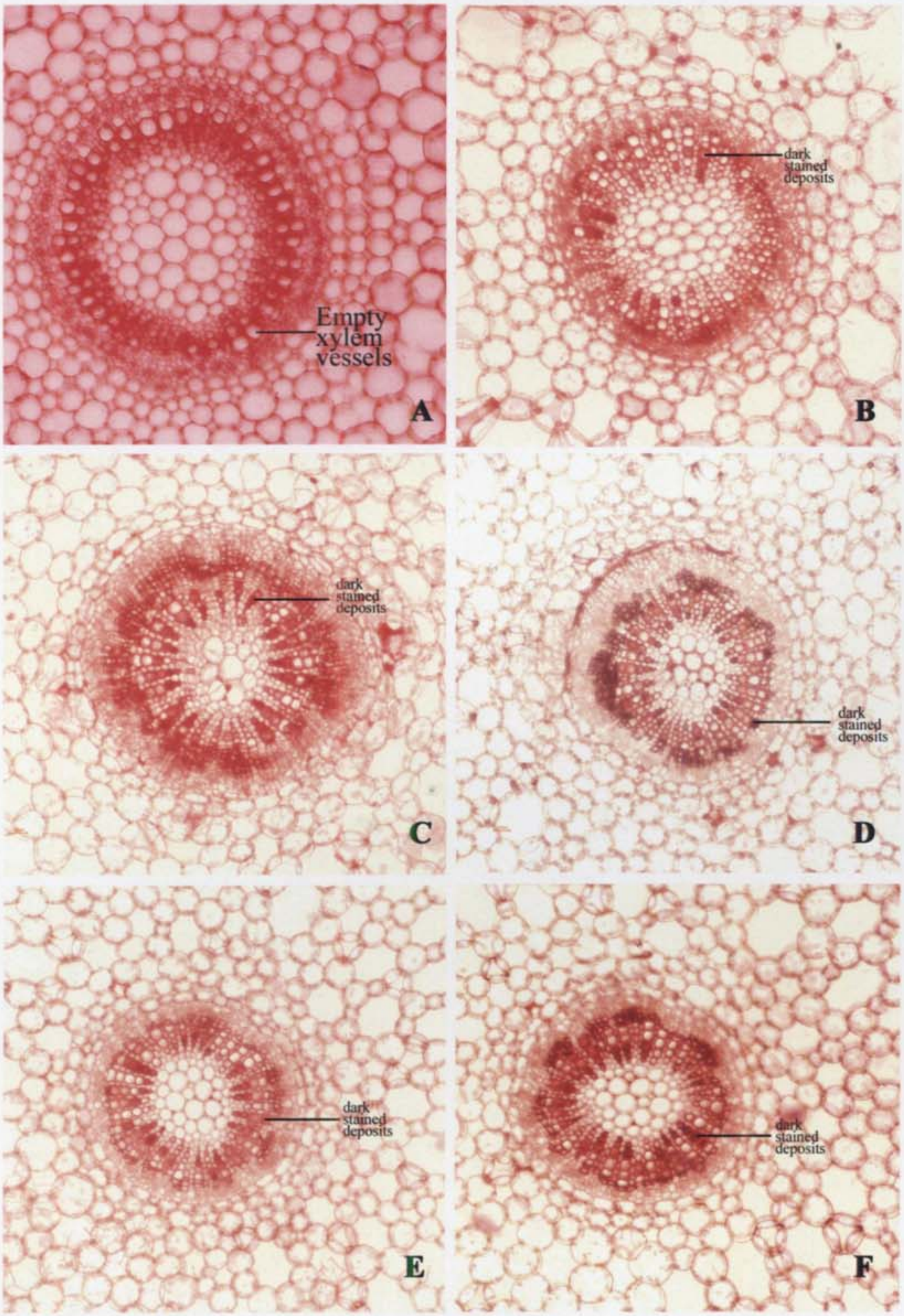

Fig. 22 Stem cross sections-*Bacopa monnieri* cultivated in various Brahmi products

A- Distilled water
B- Brahmi vita
C- Brahmi rich
D- Brahmi plus capsule
E- Jyothish Brahmi
F- Brahmi syrup

6. BIOMAGNIFICATION STUDIES

In order to test the health hazards due to the medicinal use of *Bacopa monnieri*, which is found to be an accumulator of Hg and Cd, biomagnifications study was conducted in Swiss Albino Mice by administering a known quantity of fresh plant extract and allowed to grow up to 3 weeks (Table 22). Bioaccumulation of Hg was observed in blood, brain, bone, kidney, liver, muscle, skin and hair. Maximum quantity was present in kidney followed by brain and bone. The accumulation was increased (biomagnifications) during growth as a result of repeated doses of extract given weekly.

Cadmium also showed bioaccumulation/biomagnifications in blood, brain, bone, kidney, liver, muscle and skin. Cadmium accumulation was also maximum in kidney followed by bone. Cadmium was not accumulated in hair and muscle contained negligible quantity (Table 22). The accumulation rate was progressing as a result of repeated doses of Cd administered weekly.

Mice served as controls on 0th, 7th, 14th and 21th days showed NDR levels of Hg as well as Cd in animal dry tissues and blood.

Table: 22 Biomagnifications of Mercury/ Cadmium in Swiss Albino Mice

(μgg^{-1} dry tissue)

Sampling Interval-Days						
Animal tissues	**Mercury**			**Cadmium**		
	7th	14th	21st	7th	14th	21st
Blood (**µg/ml**)	0.100	0.200	0.100	NDR	0.066	0.066
Brain	0.555	2.222	3.333	NDR	1.111	1.666
Thigh bone	0.250	0.500	1.250	1.000	1.250	2.000
Kidney	1.500	1.590	4.000	6.500	13.000	22.000
Liver	0.285	0.342	0.628	1.142	1.428	1.857
Thigh muscles	0.090	0.172	0.403	NDR	0.300	0.424
Skin	0.300	0.500	1.000	NDR	0.200	0.748
Hair	0.200	1.000	1.000	NDR	NDR	NDR
Total accumulation	3.280	6.526	11.714	8.642	17.355	28.761

NDR – Non Detectable Range

DISCUSSION

DISCUSSION

To deal with the effect of Mercury and Cadmium on *Bacopa monnieri,* a medicinal plant, screening experiments were conducted with various concentrations of $HgCl_2$ and $CdCl_2$ and confirmed that 10 µM $HgCl_2$ and 30 µM $CdCl_2$ are toxic to the plant exhibiting about 50% growth retardation. For a comparative study, plants were treated with 2, 5 and 10 µM $HgCl_2$ and 10, 20 and 30 µM $CdCl_2$. In an excellent review, Foy *et al.*, (1978) stated that sensitivity/tolerance of plants towards heavy metals vary from species to species and metal to metal. According to those authors for investigations on the effect of heavy metal toxicity, optimal concentrations are being selected based on trial experiments with various concentrations which show about 50% growth retardation. A perusal of literature revealed that treatments of 5 and 10 µM $HgCl_2$ in *Pisum sativum* (Beauford *et al.*, 1977) 25 and 50 µM $CdCl_2$ in *Bacopa monnieri* (Ali *et al.*, 1998) 10-100 µM $CdCl_2$ in *Arabidopsis thaliana* (Perfus-Barbeoch *et al.*, 2002) 1 and 2 µM $Hg(NO_3)_2$ in *Chromolaena odorata* (Velasco-Alinsug *et al.*, 2005) and 10 µM $CdCl_2$ in *Oryza sativa* (Tanaka *et al.*, 2007) were used for studies on the effect of Hg and Cd in plants.

Bacopa monnieri is a vegetatively propagated plant and hence rooted cuttings were cultivated in Hoagland nutrient solution artificially contaminated with known quantities of Mercuric chloride and Cadmium chloride. Most important visible effect of $HgCl_2$ and $CdCl_2$ treatment is inhibition of root length and shoot length (Tables 1&8). Absence of secondary root formation is another impact of both Cd and Hg. Tolerance index of both $HgCl_2$ and $CdCl_2$ treatments shows significant reduction during initial days of treatment (Tables 2&9). But after 4 days of treatment, tolerance index values are increased indicating recovery of toxic effect whereas at higher concentrations (5 and 10 µM $HgCl_2$ and 20 and 30 µM $CdCl_2$) tolerance index values are reduced throughout the experimental period (12 days). According to Wilkins (1978) primary toxic effect of heavy metals is inhibition of root growth and as the concentration of metals in the solution increases the root growth of less tolerant species is adversely affected. The author repeatedly emphasized that tolerance index is an ideal strategy to measure the degree of tolerance of plant species subjected to metal toxicity. Inhibition of root growth is reported to be one of the most important as well as rapid responses of plants to toxic concentrations of heavy metals. Wong and Bradshaw (1982) suggested that root growth is frequently used in many tolerance tests of plants.

Treatment of $HgCl_2$ at 5 and 10 µM concentrations resulted in significant increase of stomatal index in the upper epidermis (Table 3). Plants treated with $CdCl_2$ also showed almost the same pattern of stomatal index (Table 10). But more increase in stomatal index values are shown by the upper epidermal cells. Effect of heavy metals in general, Cadmium in particular on plants like *Arabidopsis thaliana* has been shown to render stomatal conductance by osmoregulation of guard cell-water relations (Perfus- Barbeoch *et al.*, 2002). According to those authors, an important aspect of Cd toxicity is perturbation of plant-water relationship. Contradictory to this, Cadmium treatment has been reported to cause stomatal closure in *Brassica rapens* (Baryla *et al.,* 2001) and increased stomatal resistance in *Brassica juncea* (Barcelo and Poschenrieder, 1990). *Bacopa monnieri* plants showed increased stomatal index on both upper and lower epidermis due to the exposal of Hg and Cd. This character may cause enhanced transpiration rate and resultant water stress. A significant role of increased stomatal index in the detoxification of Hg and Cd is apparent as it is related to the bioaccumulation pattern of these metals in *B. monnieri* which will be discussed under bioaccumulation studies.

Anatomy of *Bacopa monnieri* root and stem exhibited some changes due to the treatments with $HgCl_2$ and $CdCl_2$. Cross sections of roots appeared shrunken and granular deposits are seen all over the piliferous layer (Fig. 6a &14a) presumably due to the accumulated and/or adsorbed Hg/Cd. This observation is corroborated with the findings of Beauford *et al.*, (1977) who suggested that *Pisum sativum* and *Mentha spicata* exposed to $HgCl_2$ showed presence of Hg bound to cell wall components. Velasco-Alinsug *et al.,* (2005) reported that in *Chromolaena odorata* treated with $HgCl_2$, granular dark deposits were present on the piliferous layer of roots.

In *Phragmites australis* treated with Cd, localization of stained precipitates accumulated in parenchyma cells has been reported (Ederli *et al.*, 2004). The endodermal cells of *Bacopa monnieri* stem become conspicuous with thick walls and phloem cell's size is slightly reduced due to Hg and Cd treatments (Fig. 6b&14b). Mercuric chloride treatment seemed to stimulate suberization and hence endodermal cell wall appeared thicker in cross sections. Increased suberization and lignifications of cell walls resulting in stunted growth has been reported as one of the toxic effects of heavy metals on plants (Cseh, 2002; Ederly *et al.*, 2004; Velasco-Alinsug *et al.*, 2005).

Another important observation of heavy metal treatment is the occurrence of dark stained masses filling the lumen of xylem vessels of stem sections (Fig. 6b,c) indicating the deposits of some complexes of Mercury and/or Cadmium because such dark spots are absent in the control stem tissues (Fig. 6b,A&B). In plants subjected to Hg treatment, these deposits were confirmed to be Mercury complexes by staining the sections with dithizone which is a specific stain for the localization of Mercury (Pearse, 1972). More or less similar observations have been reported in *Chromolaena odorata* rooted cuttings treated with 1-2 µM Hg $(NO_3)_2$ (Velasco-Alinsug *et al.*, 2005). Those authors reported that root and stem sections of *C. odorata* stained with safranin showed localization of dark spots in xylem vessels. However, in *Bacopa monnieri* the dark stained deposits completely filling the lumen of the xylem (Fig. 6b,c) occur uniformly in plants treated with 5 and 10 µM $HgCl_2$ indicating high sensitivity of this plant towards mercury. However, in the case of Cd treated plants a confirmatory test for the presence of Cd is not done due to the unavailability of a specific stain. Notwithstanding, recently a specific staining method for the localization of Cd using QAI has been reported in a study of Cd treatment in *Phragmites australis* (Ederli *et al.,* 2004).

In *Bacopa monnieri* biomass reduction is very significant when subjected to 10 µM $HgCl_2$ but in the case of $CdCl_2$ treatment biomass reduction is highly significant generally in all concentration and particularly at 30 µM because only 50% dry matter was retained in this sample compared to the control after 12 days of growth (Table 4). Eventhough *B. monnieri* plant is found to be an accumulator of Hg and Cd, both heavy metals cause significant reduction of biomass (Table 4 &11). This observation is in conformity with the views of Kumar *et al.*, (1995) who suggested that although some plants can accumulate considerably high concentration of heavy metals, their growth may get significantly inhibited. According to Linger *et al.*, (2005) *Cannabis sativa* plants cultivated in soil artificially contaminated with Cd resulted in significant reduction of biomass.

In comparison with Mercury, Cadmium show more inhibitory effect on biomass production (Table 4 &11) in *Bacopa monnieri.* According to Perfus- Barbeoch *et al.*, (2002) in *Arabidopsis thaliana,* when treated with 10 μM Cd^{2+}, growth was normal due to detoxification of metals in vacuoles. But application of 20-50 μM Cd resulted in high toxicity leading to strong reduction of biomass. Cseh (2002) suggested that Cd^{2+} ions are more mobile and less stable, so occur as free ions and cause more toxic effect and the free ions may cause reduction of water potential. In this context it is obvious that accumulation potential of Cd is more compared to that of Hg. This observation is in accordance with the view of Costa and Morel (1994), who suggested that Cd is known to disturb the plant–water balance and Cd imposes decrease of water potential. The reduced water potential due to the accumulation of Cd^{2+} may stimulate water uptake from the medium and the net result is reduction of biomass as observed in *B. monnieri.* On the contrary, Hg^{2+} is not much accumulated *vis a vis* no drastic depletion of biomass occur.

Total protein content of *Bacopa monnieri* plants treated with $HgCl_2$ is reduced after 2nd day in 2 and 5 μM concentrations (Table 5). But 10 μM treatment resulted in significant reduction only 4th day onwards. Reduced protein content may be due to either inhibition of protein synthesis as reported by Reddy and Prasad (1992), unavailability of essential elements (Prasad, 1997) or inhibition of amino acid mobilization (Bishnoy *et al.*, 1993). However, protein content was gradually increased after 4 days.

Cadmium treatment also showed a reduction of protein content during 4th to 8th days and on 12th day, all treatments and control showed same quantity of protein (Table 12). Reduction of protein content has been reported in many plants due to heavy metals (Reddy and Prasad, 1992; Prasad, 1997). As a result of treatment with Cadmium, protein content was reduced in *Zea mays* (Ferretti *et al.*, 1993), sun flower (Kastori *et al.*, 1996) and barley (Stiborova *et al.,* 1986). Under heavy metal stress an 11 kDa protein was reported in green tissues (Chai *et al.*, 1998) and a 14 kDa protein in root tissues (Choi *et al.*, 1996) of bean (*Phaseolus vulgaris*) seedlings.

Poly Acrylamide Gel Electrophoretic (PAGE) studies revealed that during early days of $HgCl_2$ treatment (2nd day) there occurred the appearance of thin bands of protein with molecular weight between 47.2 – 70.4 kDa and these bands became conspicuous on 4th day (Fig. 8). During initial days (4 - 6 days) of Hg and Cd treatments of *Bacopa monnieri* the appearance of new bands of protein profile reveals the synthesis of new proteins (Phytochelatins). Several studies on Hg and Cd have been reported to induce phytochelatins synthesis for the sequestration of their toxicity (Rauser, 1987; 1990; Reddy and Prasad, 1990; Steffens, 1990; Leopold *et al.,* 1999; Kubota *et al.*, 2000; Cobbett and Goldsbrough, 2002). The appearance of new protein bands on 4th day due to heavy metal treatment in *B. monnieri* is presumed to include phytochelatins which might have synthesized before 4th day and involved in the chelation of metal ions in roots. This finding is in accordance with the view of Cobbett and Goldsbrough, (2002). According to those authors phytochelatins, thiol-rich peptides, GSH and/or cysteine-rich metallothionins are chelators to which metal ions are bound and the chelated metals in roots may be stored in vacuoles or exported to the shoots *via* xylem. However, the reduction of total protein after 4th day is not directly linked to phytochelatins. Neverthless, the overall inhibition of protein synthesis by Hg/Cd may be the cause of reduced protein content as suggested by Orcutt and Nilsen (2000), according to whom phytochelatin synthesis is a detoxification process and it should start immediately as the metal ions enter the cell, otherwise direct binding of metals to key enzymes of metabolism will lead to adverse effect. According to Leopold *et al.*, (1999) in *Silene* and tomato, phytochelatins are synthesized during 20–30 minutes after application of Cu and Cd.

As reported earlier, phytochelatins are synthesized as one of the mechanisms of heavy metal tolerance. Cadmium induced-phytochelatins are rapidly converted into a low molecular weight complex (Abrahamson *et al.*, 1992). Kneer and Zenk (1997) suggested that Cd induced phytochelatins of *Rauwolfia serpentina* undergo changes in molecular weight and these complexes enter the vacuole where they are converted into high molecular weight complex with more affinity to Cd^{2+}.

It is well known that a number of small heat shock protein-like polypeptides are induced under metal stress conditions as well as many other stresses and this induction leads to an increase in the number of denatured proteins (Sun *et al.*, 2002). According to those authors, the denaturing may be either due to strong affinity of Cd^{2+} to various functional groups of proteins or showing affinity of Hg^{2+} ions to sulfhydryl groups of proteins. However, in *Bacopa monnieri* the increase in total protein occurs during 8-12 days, whereas at earlier intervals of treatments, protein synthesis is found to be significantly reduced. In spite of the disappearance of some narrow bands, other protein bands become very prominent after 6th day contributing to total protein content as already observed in *B. monnieri* (Tables 5 &12). These observations reveal the role of phytochelatins and the sequestration of Mercury. More or less similar observations are shown by plants subjected to $CdCl_2$ treatments at 10, 20 and 30 μM concentrations (Table 12, Fig.15).

The morphological observations of *Bacopa monnieri* revealed that propagules became established and the visible symptoms of toxicity disappeared only after a minimum of 8 days which coincide with significant increase of total protein. So *B. monnieri* plants are tolerant to 10 μM and 30 μM concentrations of Hg and Cd respectively despite the manifestation of some shock or inhibitory symptoms. Another plausible reason for the reduction of proteins during initial days of Hg treatment is utilization of sulfur containing amino acids for chelation followed by sequestration to attain tolerance as reported in *Potamogeton pectinatus* (Rai *et al.,* 2003).

Chelating compounds mostly metallothionins and phytochelatins have significant role in detoxification of metals and their synthesis in the plant is induced by exposure of root cells to heavy metals (Rauser, 1999; Cobbett , 2000; Clemens, 2001; Hall, 2002; Cobbett and Goldsbrough, 2002; Rea *et al.*, 2004). These polypeptides are cysteine-rich and are able to bind with the heavy metals due to the presence of thiol groups. After chelation, the metal-chelatin complex get translocated and sequestered in the vacuoles (Clemens, 2001; Hall, 2002; Guerinot and Salt, 2001; Clemens *et al.*, 2002)

Effect of Cd on *Arabidopsis halleri* which is a mutant and hyper accumulator of Cd induces oxidative stress and results an increase in the number of misfolded protein synthesis due to short term exposure to Cd^{2+} (Weber *et al.*, 2006). In *Bacopa monnieri*, a number of thin bands (Fig. 8) were appeared due to Cd treatment in the samples of 4th day and this protein may be belonged to the misfolded protein as suggested by Weber *et al.*, (2006) in *A. halleri.* Similarly Sun *et al.*, (2002) suggested heat-shock protein induction due to Cd stress and resultant increase in protein content.

Involvement of phytochelatins in heavy metal sequestration of *Bacopa monnieri* is already known because regenerants of this plant grown under NaCl stress exhibited extra bands of 58–168 kDa protein indicating stress induced protein synthesis (Ali *et al.*, 2000).

Recently, Reverse Phase High Performance Liquid Chromatography (RP: HPLC) study by Velasco-Alinsug *et al.*, (2005) in *Chromolaena odorata* subjected to Hg treatment revealed, a prominent peak corresponding to the elution time of cystein which was similar to a phytochelatin identified as SH containing compound reported in *Rubia tinctorium* treated with Cd (Kubota *et al.*, 1995). It was also observed that treatment with higher concentration and longer periods of exposure induce synthesis of two more prominent peaks representing Hg binding peptides due to increased Hg uptake (Velasco-Alinsug *et al.*, 2005). In *Bacopa monnieri* during 8-12 days of growth, total protein as well as additional bands in PAGE occur as reported in *Chromolaena odorata.*

Distribution of total protein content of *Bacopa monnieri* plants treated with $HgCl_2$ showed significant reduction during 4–6 days and afterwards gradual increase was occurred. This observation is coincident with the reduced biomass (dry weight) content during this period in all concentrations (Table4). Eventhough *B. monnieri* accumulates considerable quantity of Hg (Table 7a, Fig. 10) and hence Hg tolerant, the plant is very sensitive initially and immediate response is shown by temporary shock and the reduced protein content may be an indirect manifestation of toxicity. Nevertheless, after 6 days the plants get recovered from the shock of toxicity and enter into the normal metabolism and protein content is increased equal to that of the control plants (Table 5&12). Increase in soluble protein content was reported in *B. monnieri* regenerants exposed to Cd and Zn (Ali *et al.*, 2000).

Chlorophyll content is often measured in order to assess the impact of environmental stress since the changes of pigments are linked with visual symptoms of growth disorder and photosynthetic productivity (Parekh, 1990). In *Bacopa monnieri* the total chlorophyll content, Chl-a, Chl-b and Chl-a/b ratio also were increased in plants treated with $HgCl_2$ and $CdCl_2$ during 8–12 days. As reported earlier, the shock of both heavy metals' toxicity was reduced during intervals (6-12 days) and so the increase in chlorophyll content may be correlated with the Hg and/or Cd toxicity. Treatment with 10 μM $HgCl_2$ resulted in significant depletion of all forms of the pigments particularly chlorophyll-b resulting in sharp increase of a/b ratio. This observation is coincident with the inhibition of chlorophyll synthesis due to Hg treatment as reported by Kupper *et al.*, (1998), Mysliwa-Kurdziel and Strzalka (2002). Inhibition of chlorophyll synthesis has been shown by many plants due to heavy metal stress. Mercury and Cadmium are reported to interact with light harvesting proteins of chlophyll of spinach leaves (Ahmed and Tajmir-Riahi, 1993). According to Oncel *et al.*, (2000) in wheat varieties treated with Cd and Pb, total chlorophyll content was decreased to 50-70% and this reduction may be result of inhibition of enzymes responsible for chlorophyll biosynthesis. Depletion of chlorophyll content in plants treated with $CdCl_2$ may be due to inhibition of chlorophyll biosynthesis (Greger and Ogren, 1991; Ferretti *et al.*, 1993) and hyperaccumulation of Cd (Stiborova *et al.*, 1986). Effect of Cd^{2+} on chlorophyll content of leaves has also been reported and this effect is strongly dependent on growth stage of the plant (Mysliwa-Kurdziel and Strzalka, 2002). According to those authors, the maximum amount of accumulated chlorophyll depended on the Cd^{2+} concentration within the range 10–50 μM and it was never as high as in untreated samples. More or less similar pattern of chlorophyll content is exhibited by *Bacopa monnieri* plants since decrease of chlorophyll content is very meagre and a trend in the increase of chlorophyll a, b and total are shown at all intervals under 10 and 20 μM concentrations of Cd.

Chlorophyll a/ b ratio can be used as a stress indicator in such a way that the ratio increases slightly with increasing concentration of $HgCl_2$ in *B. monnieri*. According to Monni *et al.*, (2001) in *Eupatorium nigrum* growing near Cu and Ni smelter, higher chlorophyll ratios were observed due to the traces of these heavy metals. Similarly increased chlorophyll ratios due to environmental stresses have been reported in spinach leaves (Delfine *et al.*, 1999).

Treatment with $CdCl_2$ in *B. monnieri* resulted in increased values of chlorophylls on second day compared to the control indicating no Cd toxicity on the pigment synthesis in all concentrations. But afterwards only gradual changes were occurred. At 20 μM concentration significant increase of chlorophyll b resulting in a sharp decline of a/b ratio was observed whereas in 30 μM, this change was not noticed. Contradictory to this observation, Zengin and Munzuroglu (2005) reported that in *Phaseolus vulgaris* seedlings treated with Cd, total chlorophyll was reduced and a/b ratio was increased. These differences may be due to variations in plant species and/or environmental conditions. Decrease of chlorophyll content is reported as one of the symptoms of Cadmium toxicity and the degree of chlorophyll decrease depends on plant species as well as the experimental conditions (Mysliwa-Kurdziel and Strzalka, 2002).

Results of bioaccumulation studies of $CdCl_2$ showed comparatively very low Cd content in leaves of *Bacopa monnieri* (Table 14a). But chlorophyll contents did not vary significantly due to Cd treatment (Table 13). The reduced Cd content of leaves may probably be a detoxification method adopted by this plant against Cd stress which is reported to be involved in the reduction of chlorophyll synthesis in *Arabidopsis thaliana* (Perfus-Barbeoch *et al.,* 2002). According to Haag-Kerwer *et al.,* (1999) in *Brassica juncea* exposed to 20-25 µM Cd, the chloroplasts are protected through a metal detoxification process by which the Cd^{2+} concentration is kept below a critical threshold level in leaves. Contradictory to this observation, Ali *et al.*, (2000) suggested that, reduction of photosynthetic rate in the Cd treated regenerants of *Bacopa monnieri* was due to disturbance in the chlorophyll synthesis.

Atomic absorption spectrophotometric studies of Hg and Cd in *Bacopa monnieri* showed accumulation of these metals in roots, stem and leaves. When cultured in Hoagland nutrient medium artificially contaminated with 5 and 10 µM $HgCl_2$, maximum quantity of Mercury was accumulated in the roots during all intervals of exposure and stem tissue contained only about half the amount of that in roots (Table 7a, Fig. 10). Mercury content of leaf was less than half that in the stem. During 12 days of growth Hg content of root was almost uniform and stem and leaves showed slight but gradual increase. When the concentration was 10 µM, Hg accumulation in the root did not change. But stem and leaves showed significant ($P<0.01$) increase in Hg content. Mercury uptake pattern of *Bacopa monnieri* is similar to that reported in *Chromolaena odorata* in which the roots accumulated the highest levels of Hg compared to the stem and leaves (Velasco-Alinsug *et al.,* 2005).

Accumulation of Hg in the roots of *Bacopa monnieri* shows a threshold level of about 40 µg g^{-1} dry tissue and this level is maintained right from the beginning (2days) to 50 days (Table 7a,b&c) of growth in Hoagland medium artificially contaminated with $HgCl_2$.

More or less uniform content of Hg is accumulated in the roots of *Bacopa monnieri* in both 5 and 10 μM $HgCl_2$ treatments (Table 7a). About 40 μg Hg g^{-1} dry tissue of roots appear to be a threshold level of accumulation to which the plants are tolerant and above this level, accumulation of Hg may cause toxicity as suggested by Beauford *et al.,* (1977). However, in the case of Cd, the accumulation is increased proportional to the concentration in the medium as well as period of growth.

Studies on bioaccumulation of Hg in plants either in natural soil or artificially contaminated media are very scanty. Similarly no plant has yet been identified as natural hyperaccumulator of Hg (Henry, 2000; Raskin and Ensley, 2000). However, transgenic plants such as *Arabidopsis thaliana, Liriodendron tulipifera,* and *Nicotiana tabacum* are capable of converting methyl mercury to Hg^{2+} and are having the potential of phytoremediation in alleviating Hg polluted areas (Bizily *et al.*, 1997; Rugh *et al.*, 1996; 1998).

According to Yoon *et al.,* (2006) a hyperaccumulator is defined as plants that can accumulate 71,000 mg kg^{-1} soil of Cu, Co, Cr, Ni or Pb. *Bacopa monnieri* plants grown in nutrient culture medium containing $HgCl_2$ accumulated 63–156 μg Hg g^{-1} dry tissue during 2–12 days of growth (Table 7b) and as per the definition put forth by Yoon *et al.*, (2006) *Bacopa monnieri* cannot be included under hyperaccumulator category. So the behavior of *B. monnieri* is in conformity with the view of Henry (2000), Raskin and Ensley (2000) who suggested that no plant is known as hyperaccumulator of Hg.

Accumulation of Cadmium also was maximum in roots. Stem contained less than half the amount of Cd that present in the root.

Cadmium content of leaf was very low. At 20 µM concentration, Cd content of root and stem was increased significantly during 12 days of growth. But leaf showed only negligible increase. As $CdCl_2$ concentration was increased to 30 µM, corresponding increase in the accumulation was not proportional to the concentration of the treatment. However, Cadmium content of leaf was increasing gradually (Table 14a, Fig. 17).

Cadmium when present in the growth medium, it is reported to be easily taken up by the roots and transported to the leaves (Siedlecka and Krupa, 1997). According to Sersen *et al.*, (2005) Maize plants grown in nutrient medium containing Cd are able to absorb it and translocate to shoot and leaves and the accumulation is proportional to the availability of the metal. The accumulation pattern of Cd in the roots of *Bacopa monnieri* is almost in consistent with the views of Sersen *et al.*, (2005) since Cd accumulation is proportional to the increase of $CdCl_2$ concentration in the nutrient medium (Table 14a,b). According to Sanita-di-Toppi and Gabbrielli (1999) immobilization of Cd by binding to the cell wall is one of the causes of Cd hyperaccumulation in plants. Enhanced accumulation of Cd in root tissues compared to shoot/leaves has been reported in many plants (Cseh, 2002). Linger *et al.,* (2005) reported that *Cannabis sativa* cultivated in soil artificially contaminated with Cd, resulted in a significant reduction of biomass and roots showed hyperaccumulation potential to absorb more than 100 mg kg^{-1} Cd in dry tissue. In *Bacopa monnieri,* inspite of the reduction of biomass (Table 11) cadmium accumulation was 458 µg g^{-1} dry tissues after 12 days of growth (Table 14a).

Cadmium also gets accumulated in the roots maintaining a threshold level, but the quantity is slightly fluctuating during prolonged period (Table 14c). However, it is evident that the metal absorbed by the root enters the xylem of root and stem and finally reach the leaves. As mentioned earlier, transverse sections of *Bacopa monnieri* root and particularly stem stained with safranin show coloured deposits filling the entire lumen of the xylem cells (Fig. 14a, b, and c). Pilon-Smits (2005) opined that the bulk flow of the metal ions from root to shoot and leaf is driven by transpiration which creates negative pressure potential in the xylem that pulls up water and solutes. As per this concept, the distribution of comparatively reduced contents of Hg and Cd is found to be due to release of these ions through stomata because bulk flow of water ions driven by transpiration pull may enable escape of the ions through stomata maintaining very low metal concentration in leaves.

Bioaccumulation, more precisely phytoaccumulation refers to the uptake and translocation of metal contaminants inclusive of essential elements in the soil by plant roots to the above ground portion of the plants (Pilon-Smits, 2005). Heavy metal contamination can be removed from water and soil using a variety of technologies and an important one is biological and mostly plants are involved in this technique so it is called phytoremediation. Due to its elegance and extent of contaminated area, phytoremediation has already received significant scientific and commercial attention (Salt *et al.*, 1998; Gleba *et al.*, 1999; Dietz and Schnoor, 2001; McGrath and Zhao, 2003; Pilon-Smits, 2005).

Aquatic plants are hyperaccumulators of several heavy metals and they are quiet effective in separating metals from their surrounding water (Lenka *et al.*, 1993; Velasco-Alinsug *et al.*, 2005; Yadav *et al.*, 2005). *Bacopa monnieri* also has been recommended as a plant for phytoremediation of metal contaminated water and wetlands (Sinha *et al.*, 1996; Sinha, 1999). Neverthless, this plant is not a hyperaccumulator of heavy metals. *Potamogeton pectinatus* is a submerged aquatic plant which is hyperaccumulator of Cd and other heavy metals and hence considered as a cost-effective phytoremediation system for cleaning of metal polluted water (Zayed *et al.,* 1998; Rai *et al.*, 1995; 2003). According to Abe *et al.,* (2006) 22 weed species growing in paddy fields are reported to be tolerant to Cd and most of them accumulate Cd in the plant body and hence could be used for phytoremediation of paddy field where soil is polluted with Cd.

In order to assess the accumulation pattern of Hg and Cd in *Bacopa monnieri*, the quantity of each metal accumulated during a specific period in relation to the availability of the same in the growth medium, separate experiments were conducted by exposing a known weight of rooted propagules to a known quantity of $HgCl_2$ and $CdCl_2$ separately during a period of 12 days and quantity of each heavy metal accumulated in root, stem and leaves were estimated separately and added together to get the total accumulation after sampling at each interval (Table 7a,b &14a,b). The residual nutrient medium was analysed to estimate the quantity of the metals left behind in the medium after sampling at each interval. In the treatment of 5 and 10 μM $HgCl_2$ and 20 and 30 μM $CdCl_2$, Hg/Cd metals were gradually decreasing during 12 days and only negligible quantities were present in the residual medium. By comparing the total Hg accumulated in the plant tissue (Tables 7b & 14b) and quantity present in the medium, it was found that obviously loss of substantial amount of the metals was occurred. The percentage distribution of Hg accumulated in plants, present in the residual medium and the calculated loss enabled to presume the release of Hg from the plant to the atmosphere. The loss (Table 7b) may occur presumably through stomata because a corresponding increase of stomatal index is shown by the plants treated with $HgCl_2$ (Table 3). Therefore *Bacopa monnieri* can be effectively used for phytoremediation to remove Hg from contaminated soil or water. According to Sinha (1999) metal accumulation property of *Bacopa monnieri* may be used for amelioration of polluted wetlands and water.

The loss/release of Hg can be considered as one of the methods of phytoremediation designated as phytovolatilization in accordance with the view of Pilon-Smits (2005), according to whom phytovolatilization is the release of pollutants by plants in volatile form. This process completely removes the pollutants from the site as gas without any need for plant harvesting and disposal. The process of volatilization can be maximised by promoting transpiration rate through sufficient irrigation. Since *Bacopa monnieri* grows profusely in aquatic environment transpir ation rate may be very high which can maximise loss of Mercury or Cadmium from the leaves. In nutrient culture also water deficit do not occur so the volatilization may be at an enhanced rate due to maximum availability of water in the medium and increased stomatal index may play an additional role in the transpiration rate of *Bacopa monnieri.*

Phytovolatilization of Selenium has been reported in plants (Terry *et al.*, 2000). Volatilization of mercury by transgenic plants (Rugh *et al.*, 1996) was achieved by introducing a bacterial mercury reductase and this plant can volatilize the elemental mercury to the atmosphere.

In the present study, loss of mercury in plants treated with 5 µM concentration of $HgCl_2$ is found to be increased proportional to the period of growth and on 12th day, more than 50% of the total quantity given is lost and at 10 µM, the corresponding loss is 59% (Table 7b). This loss can be correlated to the release of the metal either through stomata as described earlier or through trichome like appendages present all over the stem as reported in *Vigna mungo* treated with 10 µM $HgCl_2$ (Sahadevan, 2001) and in *Chromolaena odorata* treated with Hg $(NO_3)_2$ at 1 and 2 µM concentrations (Velasco-Alinsug *et al.*, 2005). The possibility of loss or release of Hg through stomata cannot be ruled out in *Bacopa monnieri,* because in plants treated with $HgCl_2$ both at 5 and 10 μM concentrations, stomatal index values were significantly higher than that of the control (Table 3) and no trichomes or similar endogenous or exogenous modifications were observed. Ali *et al.*, (2000) suggested that in *Bacopa monnieri* regenerants, Cd and Zn treatment resulted in increase of stomatal conductance. As the stomatal conductance is increased, the efflux of water through stomata also may be increased, facilitating the escape or diffusion of contaminant Mercury. Earlier, release of Hg as volatile form was reported by Siegel *et al.*, (1974) according to whom certain vascular plants accumulate Hg from soil and release as volatile form of the element from their leaves.

Another important aspect of phytoaccumulation of Hg in *Bacopa monnieri* is that the quantity of Hg given initially in nutrient medium was almost exhausted since only small quantity was retained in the residual medium after 12 days (Table 7b). In order to assess the accumulation potential of *Bacopa monnieri,* plants were allowed to grow beyond 12 days (up to 50 days) under additional dozes of $HgCl_2$. It was found that accumulation as well as loss of Hg followed the same pattern as that of 10 μM during 12 days (Table 7c), thereby confirming continuous absorption as well as release of Hg from the plants as long as the metal is present in the medium. An indirect evidence of loss of Hg from the leaves through stomata is the distribution and accumulation in the plant which range between 46% and 44% for 12 and 50 days respectively.

According to Velasco-Alinsug *et al.,* (2005) eventhough *Chromolaena odorata* plants accumulates only 1.26–2.23 μg g^{-1}, 21-23 μg g^{-1} and 30 μg g^{-1} Hg in root, stem and leaves respectively, these plants are recommended for phytoremediation because the product of Hg+Sulphur content of cysteine called 'cinnabar' is quite stable and non-degradable. Hence even after death and decay of the plant, the Hg in the form of cinnabar is stable and unavailable for further leaching into water resulting pollution. So *Chromolaena odorata* is an agent for phytoremediation by phytoextraction/ phytostabilization. On the contrary, *Bacopa monnieri* plants are able to accumulate about 90 $\mu g\ g^{-1}$ and 150 $\mu g\ g^{-1}$ Hg in 5 and 10 μM $HgCl_2$ treatment respectively (Table 7b) and the percentage distribution pattern of Hg between the plant and residual contents indicates considerable loss of Hg presumably through stomata. So the phytoremediation efficacy of *Bacopa monnieri* is manifested through phytovolatilization (Table 7b).

As mentioned earlier, bioaccumulation of Cd has been reported in many plants. Sensitivity towards accumulation of Cd in nutrient culture studies have been reported in *Typha latifolia* (Ye *et al.*, 1997) and in pea seedlings (Cohen *et al.*, 1998). According to Reid *et al.*, (2003) Cd absorbed by basal roots of potato is translocated to phloem and ultimately enters and accumulates in phloem tubes. Kruger *et al.*, (2001) suggested that divalent metals are transported as complexes of metal binding proteins within the phloem. Those authors further speculated that physical chemistry of Cd reveals the role of Cd in forming strong complexes with sulfhydryl groups of proteins as well as Cl^- ions both of which are usually abundant in the phloem. Eventhough accumulation of Cd in the shoot tissue of *Bacopa monnieri* (Table 14) and histochemical localization of dark deposits in the xylem (Fig. 14b) are important observations, deposits of metal complexes are not observed in phloem. A plausible reason for this observation may be the smaller size of the phloem cells and if at all the complexes of Cd and phloem proteins (Taiz and Zeiger, 2002) are present, the minute cells of the phloem and micro quantity of their complexes are not visible under light microscope. Neverthless localization of Cd complexes is clearly observed in the xylem vessels of Cd treated plants whereas in control plants xylem vessels appear empty (Fig. 14b, A&B). Anatomy of tissues showed thick walled cells constituting the outermost piliferous layer of roots and dark deposits in all xylem vessels of *Bacopa monnieri* stem (Fig. 6a,b andc). A comparable result was reported in *Phragmites australis* in which dark brown deposits (stained with safranin) were observed in stem and root cells as a result of Cd treatment (Ederli *et al.*, 2004).

As mentioned earlier, Cd^{2+} are fast mobile in plants. Many plants such as *Potamogeton pectinatus* (Rai *et al.*, 2003), *Arabidopsis thaliana* (Perfus-Barbeoch *et al.*, 2002), *Phragmites australis* (Ederli *et al.*, 2004), *Brassica juncea* (Ishikawa *et al.*, 2006) are reported as hyperaccumulators of Cd. Eventhough most of the Cd accumulators are recommended for phytoremediation (Pilon-Smits, 2005), translocation of Cd^{2+} to rice grains causing health hazards have been reported recently by Tanaka *et al.*, (2007). According to those authors, Cd^{2+} are transported to *Oryza sativa* grains through phloem and about 90-100% of the Cd is present in phloem sap.

In *B. monnieri* storage tissues/organs are not present as it is a vegetatively propagated herb and hence the phytovolatilization of accumulated Hg and Cd is the only mode of detoxification which is essential for absorption /translocation of these metals continuously from the medium.

Yoon *et al.*, (2006) proposed a specific pattern or scheme to assess the ability of plants to accumulate metals from soil. According to those authors bioconcentration factor (BCF) is defined as the ratio of metal concentration in the roots to that of soil and the plants' ability to translocate metal from the root to the shoot is named as translocation factor (TF) which is defined as the ratio of metal concentration in the shoot to root. By comparing BCF and TF, the ability of different plants for metal absorption and translocation to the shoot can be assessed. Tolerant plants restrict heavy metal translocation from soil to root and root to shoot which result in less accumulation, while hyperaccumulators actively take up and translocate heavy metal to the above ground biomass. According to Fitz and Wenzel (2002), plants exhibiting TF and BCF values less than one are unsuitable for phytoextraction.

Bacopa monnieri plants grown in nutrient medium containing known quantity of $HgCl_2$, exhibited accumulation of Hg in root and shoot. By considering the quantity of Hg accumulated in the root and shoot and that present in the nutrient solution after growth for a period of 12 days, the ratio of BCF and TF (Yoon *et al.*, 2006) is applied to assess the hyperaccumulation character. At 5 µM concentration of $HgCl_2$, the BCF values are above one after 4 days whereas 10 µM concentration shows BCF value less than one (Table 14d). Reduced BCF values in 10 µM $HgCl_2$ indicate the limited absorption during the short period of 4 days. The pattern of TF ratio is just reverse in the case of 10 µM $HgCl_2$ treatment which shows more TF ratio because the accumulation potential of root is almost constant irrespective of the amount translocated to the shoot which is getting increased progressively during 12 days. Another reason for comparatively reduced value of TF ratio in both Hg and Cd is seemed to be significant loss of these metals from the shoot through stomata as described earlier and this loss is reflected in lowering the TF ratio since it is an expression of relative of accumulation of the metals in shoot and root. When the growth period was extended to 50 days with additional dozes of $HgCl_2/CdCl_2$, the BCF and TF ratios of Hg and Cd accumulation were almost similar to the observations of 5 and 10 μM $HgCl_2$ during 12 days of growth and to the residual quantity of these metals (growth medium) were also considered (Table 14d).

In the case of Cd, the BCF ratio of 20 μM and 30 μM $CdCl_2$ is more or less similar to $HgCl_2$ treatment except the values of 10 μM treatment on 10th and 12th days which are more than one revealing more accumulation potential of Cd compared to Hg during those intervals (Table 14 d). The TF values of Cd accumulation in both concentrations show less than one, registering more accumulation in the root tissue compared to the shoot and the Cd content in the root is progressively increased proportional to the period maintaining the ratio less than one.

The lowering of TF ratio in the concentration of Hg and Cd accumulation in *Bacopa monnieri* confirms that the mode of phytoremediation is phytovolatililzation. Volatilization removes the pollutant almost completely from the site without need for plant harvesting and disposal and this process can be considered as an attractive technology as suggested by Pilon-Smits (2005).

Accumulation of Hg and Cd in *Bacopa monnieri* plants grown hydroponically is dependent on the presence of other ions as well as the pH of the growth medium. The plants cultivated in distilled water containing equal quantities of $HgCl_2$ and $CdCl_2$ (10 μM) having the pH-5.5 showed a slight increase in accumulation of Hg (Table 15). The accumulation of Cd was 8 times higher. A more or less similar observation was reported in *Helianthus* and *Hibiscus* where a doubling of

Cd uptake was occurred by the application of Cl^- to reduce the soil pH (Hattori *et al.*, 2006). The behaviour of *Bacopa monnieri* towards pH changes of soil in the absorption mode of Cd is in accordance with the findings of Hattori *et al.*, (2006). However, at basic pH (7.5) Hg accumulation was slightly reduced compared to individual treatments at pH-6.8 and 5.5 whereas Cd was reduced exorbitantly in comparison with the pH-5.5 and significantly compared to pH-6.8 (Table 15). This may be due to the flux and/antagonistic effect of Ca^{2+} in the medium. Similar results were reported in wheat (Hough *et al.*, 2003) in which the uptake is depending on soil pH and liming to pH-7 will reduce Cd concentration in wheat grains.

According to McBride *et al.*, (1997) bioavailability (plant uptake or toxicity) of metals is associated with solubility and is limited with pH of the soil. Bioaccumulation of Cd in plants depends upon soil characters such as soluble concentration, pH, organic matter and plant species (Sauve *et al.*, 2000; Podar *et al.*, 2004). As described earlier, when the pH is increased to 7.5 by adding calcium hydroxide, Cd accumulation was exorbitantly reduced in *Bacopa monnieri* (Table 15). The abundance of Ca^{2+} in the medium due to the addition of calcium hydroxide may play an indirect role in limiting the entry of Cd^{2+}. According to Kim *et al.*, (2002) Ca^{2+} inhibit Cd accumulation in rice roots. Those authors suggested that Cd^{2+} may substitute Ca^{2+} and hence Ca^{2+} concentration alters Cd accumulation. Conversely, in the case of low Ca^{+} concentration, much more Cd^{2+} is translocated to the shoot compared to high concentration where most of the Ca^{2+} remains in the shoot (Skorzynska-Polit *et al.*, 1998). Perfus-Barbeoch *et al.*, (2002) suggested that in *Arabidopsis thaliana*, $CdCl_2$ induced stomatal closure by controlling the closing/opening of Ca^{2+} channels of plasma membrane of guard cells and Cd^{2+} mimics Ca^{2+}

channels and enter guard cell through voltage dependent Ca^{2+} channels. According to Reid *et al.,* (2003) Cd accumulation in plants is due to many physical similarities between Ca^{2+} and Cd^{2+}. The ionic radius of Ca^{2+} (99 pm) and that of Cd^{2+} (97 pm) causes sharing of Ca^{2+} channel with Cd^{2+} for the absorption and translocation. In potato tuber it has been reported that Cd^{2+} follows the route of Ca^{2+} for translocation (Reid *et al.*, 2003). Neverthless, unlike Ca^{2+}, Cd^{2+} ions show high affinity towards sulfhydryls for binding or complexation with phloem constituents.

On the whole, the result of increased pH due to addition of $Ca(OH)_2$ to nutrient medium results in the abundance of Ca^{2+} which controls the entry of Cd^{2+} through Ca^{2+} channels. In this context, 'liming' can be recommended as a measure against Cd^{2+} absorption and translocation in *Bacopa monnieri.* Since it is a medicinal plant, the accumulation of Cd causes serious health hazard and is obviously dangerous to mankind. On the contrary, reduced pH of the growth medium (5.5) by adding NH_4Cl results in eight fold increase of Cd uptake (Table 15) and this behaviour of *Bacopa monnieri* can be exploited for phytoremediation technology for purification of water and soil contaminated with Cd.

Analysis of growth and bioaccumulation of Mercury and Cadmium in *Bacopa monnieri* plants cultivated in Hoagland medium contaminated with known quantities of heavy metals reveal the absorption and accumulation of Hg and Cd in roots, stem and leaves. Effect of acidic pH (5.5) and basic pH (7.5) showed increased and decreased Cd uptake respectively and the former quality can be recommended for phytoremediation while the latter prevents the health hazard due to the medicinal use of this plant.

Loss of both Hg and Cd from the leaves to the atmosphere is another method to reduce the bioaccumulation of these elements. Hence it is interesting to note that some sort of 'cycling' of Mercury and Cadmium occur from the nutrient medium to the plant and from the plant to the atmosphere. So *Bacopa monnieri* plant is neither an excluder (Baker, 1981; Fitter and Hay, 1983; Baker and Walker, 1990; Turner, 1994; Salt *et al.,* 1998; Orcutt and Nilsen, 2000; Pilon-Smits, 2005) nor an accumulator of Mercury (Baker and Walker, 1990; Turner, 1994). Similarly, this plant never shows strategies like avoidance, internal detoxification or biochemical tolerance (Berry, 1986) because both Hg and Cd enter the plant and internal detoxification or biochemical tolerance are not apparent. However, the strategies of response shown by *Bacopa monnieri* towards Mercury and Cadmium may be considered as amelioration. According to Fitter and Hay (1983), amelioration means plant absorb the toxic ions and act upon it in such a way as to minimize the effect variously and this may involve chelation, dilution, localization or excretion. The 'cycling' of Mercury and Cadmium between growth media and atmosphere involves absorption, chelation to some extent by phytochelatins formation, localization in roots, translocation to the shoot and finally 'excretion' through stomata. If the growth medium is soil contaminated with Hg/Cd (heavy metals) the metals may enter the plant, translocates to the leaves and gets returned to the atmosphere, and finally reach the soil from the atmosphere. So the 'cycling'of Mercury and Cadmium here can be considered as Soil-Plant-Atmosphere-Continuum, comparable to the SPAC concept of Water Relations in plants.

Bacopa monnieri plant is found to be very sensitive to water pollution, since the plants grown in different water samples collected from different areas near by Calicut University Campus and distilled water artificially contaminated with $HgCl_2$ showed dark colored deposits in the xylem vessels when stained with safranin (Fig. 18 a,b). Localization of Mercury was confirmed by staining sections with dithizone (Pearse, 1972) as shown in figure (6c, I&J).

Having obtained the results of high sensitivity of *B. monnieri* towards water pollution, a study was conducted to analyze the presence of heavy metals in the plants growing under natural habitats of some selected regions of Kerala (Table 16). So it is inferred that *B. monnieri* plants growing in majority of natural habitats shown accumulation of heavy metals and /or some other contaminants of polluted water/soil.

Based on these observations of high accumulation potential and sensitivity of *Bacopa monnieri* to water pollution and/or to micro quantities of heavy metals, rooted propagules were grown in different local water bodies (Table 17 A&B), commercially available mineral waters (Table 18 A&B), different soft drinks (Table 19 A&B), fruit juices (Table 20 A&B) and 'Brahmi' products (Table 21 A&B) which are believed to contain traces of heavy metals. The observations obtained are very interesting and the plants showed absorption and translocation of various metals present in the above solutions inclusive of essential heavy metals like Cu, Fe, Mn, Zn and non-essential heavy metals like Al, As, Cd, Cr, Pb, etc.

When comparison is made between the heavy metals present in the medium (local waters, mineral waters, soft drinks, fruit juices and Brahmi products) and the quantity of the metals entered *Bacopa monnieri* when cultured in those solutions during a period of 4 days, it was observed that all metals present in the medium were absorbed by the plant. The accumulation pattern of metals expressed as concentration (Table 17A, 18A, 19A, 20A, and 21A) and content (Table 17B, 18B, 19B, 20B and 21B) are very significant.

Histological staining of free-hand sections of stem tissue of plants grown in the above solutions stained with safranin showed the presence of heavy metals (and other contaminants also) appeared as dark stained deposits filling almost all xylem vessels and some spots in the cortex and pith region also (Fig. 18 a,b; 19, 20 a,b; 21, 22). This observation indicates the high sensitivity of *B. monnieri* towards contamination of growth medium and hence this plant can be used for monitoring water/soil pollution.

Eventhough safranin is not a specific stain for heavy metals, densely stained spots are localized inside the vessels, cortex and pith parenchyma. Localization of Hg has been reported after staining with safranin in the cross sections of root, stem and leaves of *Chromolaena odorata* treated with Hg $(NO_3)_2$ by Velasco-Alinsug *et al.*, (2005). As described earlier, the present author also stained the stem cross sections of Hg treated plants with safranin and similar dark deposits were observed (Fig. 6b and c) and using dithizone (Pearse, 1972), the localization of Hg was confirmed (Fig. 6c, I&J). It is surprising to observe more or less similar colored deposits in the stem cross sections of *B. monnieri* treated with $CdCl_2$ (Fig. 6c, K&L), mineral waters, soft drinks, fruit juices and Brahmi products. So the specificity of this staining method is not specific for Hg. Nevertheless, in order to demonstrate the contamination of water or any other solution or medium to which the plant is exposed, *Bacopa monnieri* is an ideal material as this plant is highly sensitive to micro quantities of each and every elements.

The quantity of each metal accumulated in the plant body is proportional to the amount present in the medium except some essential metals such as Cu, Fe, Mn and Zn (Table 18A). This may be due to antagonism between essential elements or with non-essential heavy metals. Antagonism and synergism have been observed in plants, but antagonism is more common (Kabata-Pendias, 1992 ; Orcutt and Nilsen 2000).

The antagonism is clearly observed in the case of mineral water (Aquaspring) where the accumulation of essential elements such as Cu, Fe and Zn are comparatively low due to the antagonistic effect of Ni (Table 18 B), as suggested by Orcutt and Nilsen (2000). Synergistic effect is also observed in the distribution pattern of some metals in the bioaccumulation in *Bacopa monnieri* from mineral water as shown in 'Omkar' mineral water, where synergism is shown by Zn, Ni, Fe, Cu and Cd.

The present study reveals that when *Bacopa monnieri* exposed to very low concentration of $HgCl_2$ and $CdCl_2$, major quantities of these heavy metals are accumulated in all plant parts and the pattern of accumulation is Root>Stem>Leaf. Since *B. monnieri* is an emergent aquatic species, it is often exposed to contaminated water and soil of wetlands, which are used for anthropogenic and industrial waste disposal. *Bacopa monnieri* is a commonly used and highly consumed multipurpose medicinal plant (Nair, 1987; Sivarajan and Balachandran, 1994; Bhattacharya *et al.*, 2001; Chakravarthy *et al.,* 2001; 2003; Russo and Borrelli, 2005) and this plant has also been reported and recommended as an agent for phytoremediation since it is an accumulator of many toxic heavy metals such as Hg, Cu, Cd, Cr, Mn and Pb (Sinha *et al.*, 1996, Sinha, 1999). This paradoxical behaviour of *Bacopa monnieri* is to be taken into consideration when large scale cultivation is done for commercial purposes. Indiscriminate cultivation and/or collection of materials from contaminated waste water may lead to serious health problems, as this plant is widely used for many Ayurvedic preparations and food supplements.

Now a days pharmaceutical and nutraceutical use of *Bacopa monnieri* are fast increasing because people are crazy over the consumption of Ayurvedic medicines and food supplements containing *Bacopa monnieri* which are supposed to increase the memory power.

In order to assess the gravity of health hazard, due to the consumption of *Bacopa monnieri* plants containing toxic heavy metals, the author conducted some experiments to test the biomagnification effect by using the plants grown in Hoagland solution artificially contaminated with known quantities of $HgCl_2$ and $CdCl_2$. Fresh aqueous homogenate of these treated plants was administered intragastrically to healthy Swiss Albino Mice and accumulation of Hg and Cd in the body of animal was detected. It is very interesting to note that even when very small quantity of plant homogenate containing 4.8–14.4 µg of Mercury was administered at three intervals, accumulation of Hg was detected in the animal tissues at the range of 3.28–11.71 µg, distributing in almost all internal organs and the concentration of accumulation was increased proportional to the repeated administration of plant homogenate contaminated with Hg (Table 22). In the case of Cadmium, accumulation/biomagnifications rate was different in such a way that when Cd content of 33.9–101.7 µg was administered, accumulation was detected in the range of 8.64–28.76 µg/animal organs/tissue, distributing in almost all internal organs and the accumulation was increased proportional to the repeated administration of plant homogenate contaminated with Cd.

Mice administered with *Bacopa monnieri* aqueous homogenate showed accumulation of both Hg and Cd in various organs. Maximum Mercury content was in kidney followed by brain and bone where as Cadmium content was maximum in kidney followed by bone and brain. In hair, Cd accumulation was absent. In both the cases minimum accumulation was detected in muscles and blood (Table 22).

SUMMARY AND CONCLUSIONS

Hussain K. “Ecophysiological aspects of bacopa monnieri (l.) pennell” Thesis.
Department of Botany, University of Calicut, 2007

SUMMARY AND CONCLUSIONS

Investigations are made for elucidating toxic effects of different concentrations *i.e.*, 2, 5, 10 μM $HgCl_2$ and 10, 20 and 30 μM $CdCl_2$ on *Bacopa monnieri* rooted propagules, cultured in Hoagland nutrient medium under natural environmental conditions. Parameters of toxicity assessment include growth performance of root and shoot, tolerance index, stomatal index, total protein content, PAGE profile of proteins and chlorophyll analyses. Bioaccumulation study of Hg and Cd in root, stem and leaves by Atomic Absorption Spectrophotometry also included to elucidate the mode of absorption and translocation and to examine the phytoremediation efficacy of *Bacopa monnieri.* Histochemical staining of root, stem and leaf cross sections, are done for confirmation of localization of the heavy metals in various tissues.

In order to confirm the sensitivity and bioaccumulation strategy of *Bacopa monnieri* towards heavy metals, rooted propagules were cultivated in water samples collected from various polluted areas, commercially available mineral water, soft drinks, fruit juices and Brahmi products. Biomagnifications study was conducted on white mice by administering plant extract containing $HgCl_2$ and $CdCl_2$.

The main observations and conclusions extractable from the data are *Bacopa monnieri* plants are highly sensitive to very low concentrations of $HgCl_2$ and $CdCl_2$. Toxic effects are detected at the concentrations of 5 and 10 μM of $HgCl_2$, 20 and 30 μM of $CdCl_2$ and about 50% growth retardation was exhibited by the plants at 10 μM $HgCl_2$ and 30 μM $CdCl_2$. Due to $HgCl_2$ and $CdCl_2$ treatments, inhibition of root length and shoot length were significant. Tolerance index and stomatal index value were gradually increased proportional to the concentration of heavy metal treatment.

Toxicity of these metals is not significantly exhibited in the distribution of total protein and chlorophyll contents, in spite of the occurrence of the new protein bands which are presumed to be phytochelatins.

Histochemical localization of Hg and Cd showed stained deposits along the piliferous layer and elongated contaminant patches in undifferentiated vascular tissues of roots and stained deposits completely filling the lumen of almost all xylem vessels of stem. Leaves did not show such stained deposits.

Bioaccumulation of Hg and Cd was in the pattern of R >S >L. Disparity was observed in the distribution of both metals when comparison was made between the quantity given in the treatment and content accumulated in the plant (root, stem and leaf together). A significant loss of both metals was observed. The loss is presumed to be due to the release of the metals from shoot probably through stomata which was found increased in number on both upper and lower epidermis. Increased stomatal index values of both lower and upper epidermis are found to be related to the loss of metal from the leaf, plausibly by phytovolatilization which is an important mode of phytoremediation strategy shown by plants.

Bioaccumulation studies show translocation of the metals to the entire plant body. Distribution pattern of both metals in different plant parts and metals retained in the medium after 12 days of growth, revealed the loss of significant amount of metals from the plant. The same pattern of absorption, translocation and phytovolatilization was confirmed by growing *Bacopa monnieri* plants in nutrient medium containing $HgCl_2$ and $CdCl_2$ for a period of 50 days.

Conclusions:

1) Eventhough *Bacopa monnieri* plants are sensitive to very low concentrations of Hg and Cd and about 50% growth retardation is exhibited in 10 μM $HgCl_2$ and 30 μM $CdCl_2$ the plants survive in the growth media.

2) Transient growth inhibition occurs up to 6-8 days showing temporary shock and normal growth is regained soon after.

3) Physiological parameters such as distribution of chlorophylls, total protein, and PAGE profile of proteins are not much changed due to the heavy metals. However, PAGE profile showed some new bands of phytochelatins which appear as a means of metal detoxification.

4) *Bacopa monnieri* shows bioaccumulation potential of both Hg and Cd in root, stem and leaf in the pattern of R>S>L.

5) The phytoremediation potential of *Bacopa monnieri* is significant, eventhough considerable loss of both heavy metals assumed from the calculations of the quantity of metals supplied, left behind in the residual medium and accumulated in the plant body occurs. This loss is presumed to be due to phytovolatilization from the leaves through stomata.

6) When Bioconcentration factor (BCF) and Translocation factor (TF) of both Hg and Cd are correlated, the values are less than one in the case of both metals, though the shoot system accumulates more Hg and Cd. The loss of these metals from the shoot system by phytovolatilization results in the lowering of the ratios and hence

Bacopa monnieri can not be included under hyperaccumulator of Hg or Cd.

7) Comparison between distribution and accumulation of Hg and Cd present in the residual medium and loss occurred during growth for 12 days, show more or less uniform quantity of Hg and Cd is retained in the plant. But in the medium, the metals are almost exhausted as growth proceeded and loss is proportionally increased.

8) When additional doze of heavy metals is given and growth proceeded up to 50 days also, the quantity of both Hg and Cd accumulated in the plant body maintained more or less uniform quantity and loss was proportionally increased as growth advanced.

9) The distribution pattern of Hg and Cd in the plant, nutrient medium and loss occurred from the leaves exhibits some sort of 'cycling' of these metals and hence a continuum is established between soil, plant and atmosphere comparable to the SPAC concept of water relations in plants.

10) Absorption and accumulation of Hg and Cd is maximum at acidic pH and very low at alkaline pH. So, for medicinal purpose, cultivation in alkaline soil/water and for phytoremediation purpose, cultivation in acidic soil/water is recommended.

11) Cultivation of *Bacopa monnieri* in water samples such as tap water, sewage water, rain water etc. confirmed the sensitivity of this plant towards pollution by microscopic observations of stem tissue which showed accumulation of stained deposits in the xylem and due to this sensitivity it can be used as a plant for 'monitoring' water pollution.

12) Sensitivity of *Bacopa monnieri* towards heavy metal pollution is confirmed by absorption and accumulation of various heavy metal contaminants (Al, As, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb and Zn) when rooted propagules were cultivated in commercially available mineral water, soft drinks, fruit juices and Brahmi products.

13) The medicinal property and wide use of *Bacopa monnieri* as an ingredient of many Ayurvedic medicines and food supplements on one hand and bioaccumulation potential and phytoremediation efficacy on the other are paradoxical.

14) Biomagnifications study on white mice resulted in the accumulation of these metals in various essential organs of the animal, confirming the gravity of health hazard due to the consumption of even a very low quantity of contaminated *Bacopa monnieri* plant tissue.

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